REPRODUCTION IN FATHEAD MINNOWS *(PIMEPHALES PROMELAS)* FOLLOWING WATER OR SEDIMENT EXPOSURE TO A COMPLEX URANIUM MILLING EFFLUENT ELEVATED IN SELENIUM

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By: Melissa Kay Driessnack

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ABSTRACT

Northern Saskatchewan, Canada is home to a uranium milling operation that discharges a complex effluent containing elevated concentrations of nutrients, compounds and many metals including the metalloid selenium (Se). Selenium has the potential to bioaccumulate in aquatic systems even when water concentrations are low, which has resulted in the identification of Se as a constituent of concern in affected aquatic ecosystems. This research evaluated the effects of treated uranium milling effluent and contaminated sediment in isolation and in combination to determine the contribution and importance of each source to fathead minnow (*Pimephales promelas*) reproduction and survival. Identification of pathways of exposure is critical to the mitigation of any observed effects. Trios of fathead minnows were allocated to one of four treatments for 21-days where the following were evaluated: survival (adult and 5 day larval), larval deformities, reproductive effects (egg production, spawning events) and metal tissue burdens (muscle, ovary, eggs and larvae). In addition, Se speciation analysis was conducted on selected fish tissues. Effects were overwhelmingly effluent-mediated with little contribution observed due to the presence of contaminated sediments. The contaminated sediments tested were taken from the actual receiving environment and represented the sediment composition found in greatest abundance in areas characteristic of fathead minnow habitats. Results showed egg production significantly increased in the effluent treatments compared to the reference water treatments ($p \leq 0.05$). Although egg production increased following effluent exposure, there was reduced hatching success by 23% ($p = 0.001$), and larval survival by 31% ($p = 0.001$) and a significant increase in skeletal deformities (i.e. scoliosis, lordosis) in 5 day old larvae by approximately 6-fold ($p = 0.001$) relative to control. Despite these effects on the offspring, when examined in an integrated manner relative to increased egg production, the mean number of normal larvae did not differ among treatments ($p > 0.05$) when compared to reference water treatments. Many metals including total Se and Rb significantly increased ($p \leq 0.05$) in the effluent exposed algae/biofilm collected from the streams, eggs, larvae and female muscle and gonads. A shift in the speciation of Se was evident with exposure, where for the larvae selenocystine-like compounds were found to be around 80% and selenite at 5-11% of the total Se in reference water exposed larvae. However, in effluent exposed larvae selenocystine-like compounds dropped to 53-68% and selenomethionine-like compounds increased to about 28%. The algae/biofilm present in the mesocosms was identified as key in the transfer of available Se

into the food chain from the water and was a source of direct dietary exposure to fish and possibly invertebrates. Additional analysis of the data was carried out using correlation and Principal Component Analysis (PCA) techniques. The results there showed a strong correlation between Se in water and algae, as well as among Se in the ovary and eggs and larvae. Correlation work was also done with Rb and strong correlations were noted between Rb in and eggs/female/day. Principal Component Analysis results showed the loading of other metals, such as Zn, Cd, and As loaded more strongly in the first component compared to Se in the third component. A summary of all the results indicate that exposure to an environmentally relevant concentration (25%) of uranium milling effluent leads to increased egg production, yet the resulting eggs and larvae experienced reduced hatching success, survival and increased incidence of deformities. While much of the focus of the initial work was on Se in the environment and fish tissues the later correlation and PCA work suggest that Se is contributor to the system but not the sole causal factor of all observed results.

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So this is probably take three at an appropriate acknowledgement and then I realized the number of people who deserve thanks for not only helping on this research but helping me grow as an individual is huge and I can't help but smile at how lucky I am. I grew up with an amazing family and thinking of them reminds me of an award that was in my Dad's office that said "It takes a village to raise a child" and I sit here now thinking how accurate it is. I was guided by parents who made sure I was in positions where I could learn everything from academics to life skills by exceptional groups of people. And though there have been many challenges faced during my time on this project I am grateful to everyone who helped see me through even when I couldn't. I have probably learned more in these last 3 years then my previous 23. So thank you to my supervisor Dr. Monique Dubé, for her willingness to take me on as a student and her continued support provided throughout this project. She has taught me more than how to be scientist but also how to stand strong and confident on my own. To Dr. Paul Jones with your help I have been able to realize and begin to achieve my personal and professional goals, there is no way to thank you enough. As well thanks to my committee members Dr. David Janz and Dr. Barry Blakley. My upmost and deepest respect and gratitude to all of you.

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"I believe that everything happens for a reason. People change so that you can learn to let go, things go wrong so that you appreciate them when they're right, you believe lies so you eventually learn to trust yourself, and sometimes good things fall apart so better things can fall together."
— Marilyn Monroe

"Remember, if you ever need a helping hand, it's at the end of your arm, as you get older, remember you have another hand…to help others."
— Audrey Hepburn

LIST OF ABBREVIATIONS

25% – 25% diluted uranium milling effluent

ANOVA– Analysis of Variance

CCME – Canadian Council of Ministers of the Environment

CLS – Canadian Light Source

CF – condition factor

CH_3SeH – methylselenol

DMSeO – dimethylselenoxide

d.w. – dry weight

EWRS – 25% Diluted Effluent: Reference Sediment

EWCS – 25% Diluted Effluent: Contaminated Sediment

e/f/d – eggs/female/day

EEM – Environmental Effects Monitoring program of Environment Canada

FHM – fathead minnow

GSe – seleno-diglutathione

GSI – gonadosomatic index

HXMA – Hard X-ray Micro Analysis

HPLC – High Pressure/Performance Liquid Chromatography

ICP-MS – inductively coupled plasma mass spectrometry

IC-ICP-MS –Ion chromatography coupled - plasma mass spectrometry

IOC – Investigation of Cause

K-S Test – Kolmogorov-Smirnoff Test

LSI – liver somatic index

MMER – Metal Mining Effluent Regulation

MSe – metal selenide

NH_3-N – ammonia

$0_2^{\bullet-}$ – superoxide anion

OECD – Organization of Economic Cooperation and Development

ROS – Reactive oxygen species

R-Se-Se-R – selenocystine-like compounds

R-Se-R – selenomethionine-like compounds

RWRS – Reference Water: Reference Sediment

RWCS – Reference Water: Contaminated Sediment

SRC – Saskatchewan Research Council

TOC – Total Organic Carbon

TSS – Total suspended solids

US EPA – United States Environmental Protection Agency

XAS – X-ray absorption spectroscopy

XANES – X-ray absorption near-edge spectrum

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PREFACE

This thesis has been written in manuscript format. Chapter 2 is a manuscript published in Ecotoxicology. Driessnack MK, Dubé MG, Rozon-Ramilo L, Pollock R, Jones PD, Pickering IJ, Wiramanaden C (2011) The use of field-based mesocosm systems to assess the effects of uranium milling effluent on fathead minnow *(Pimephales promelas*) reproduction. *Ecotoxicology* 20: 1209-1224. This has led to some repetition in the introduction and conclusions in the thesis. Chapter 3 will be submitted to the Journal of Integrated Environmental Assessment and Management under joint authorship with Dubé MG and Jones PD.

CHAPTER 1:
General Introduction

1.1 Uranium Mining

Saskatchewan, Canada is home to two of the world leaders (Cameco Corporation and AREVA Resources Canada) in uranium production for use in nuclear energy generation. Northern Saskatchewan has been the major source of Canadian uranium mining starting with activities at Beaverlodge Mine in 1953. Presently there are three active mines (McArthur River, McClean Lake and Rabbit Lake) and three milling sites (Key Lake, McClean Lake and Rabbit Lake) in Saskatchewan (Figure 1.1). Other uranium deposits have been identified and are currently in various stages of exploration and development such as the Millennium Deposit and Cigar Lake. Other sites include Kintyre in Australia, Smith Ranch Highland, Wyoming, Crowe Butte, Nebraska, and Inkai, Kazakhstan where Cameco Corp holds ownership (www.cameco.com, www.cna.ca). Uranium production can be described as a two phase process consisting of mining the rock ore and then a subsequent milling and enrichment process. Mining involves the extraction of the uranium-containing ore from the ground while milling is a multistep process in which the uranium is extracted from the ore to produce a concentrate known as yellowcake (U_3O_8). The yellowcake is later refined and enriched for use in energy production. Refining does not occur at the mine or milling sites in Saskatchewan. In 2010, alone the Key Lake and McArthur River milling facilities produced 13.9 million lbs of yellowcake (www.cameco.com). The Key Lake facility is a former open pit mine located in north-central Saskatchewan (57°11'N, 105°34'W), approximately 600 km north of Saskatoon, SK, Canada. The active mining of the Gaertner ore body was completed in 1987 and the Deilmann ore body was completed in 1997. This has led to a transition from mining to milling at the Key Lake facility which is currently the primary activity at the site (Klaverkamp et al. 2002; Pyle et al. 2002a). Milling activities are expected to continue for the next 2 to 3 decades as the facility continues the processing of ore from the McArthur River Mine and potentially from the Millennium Deposit (CNSC 2006, www.cameco.com).

As of 2007, the milling site at Key Lake has been approved to produce 7.2 million kg of uranium annually (Cameco Annual Report 2007). The production of yellowcake results in the generation of a milling effluent that is released to the environment via Wolf Lake. In 2007, 1,552,868 m^3 of effluent was released. The reference site for Key Lake is primarily David Lake

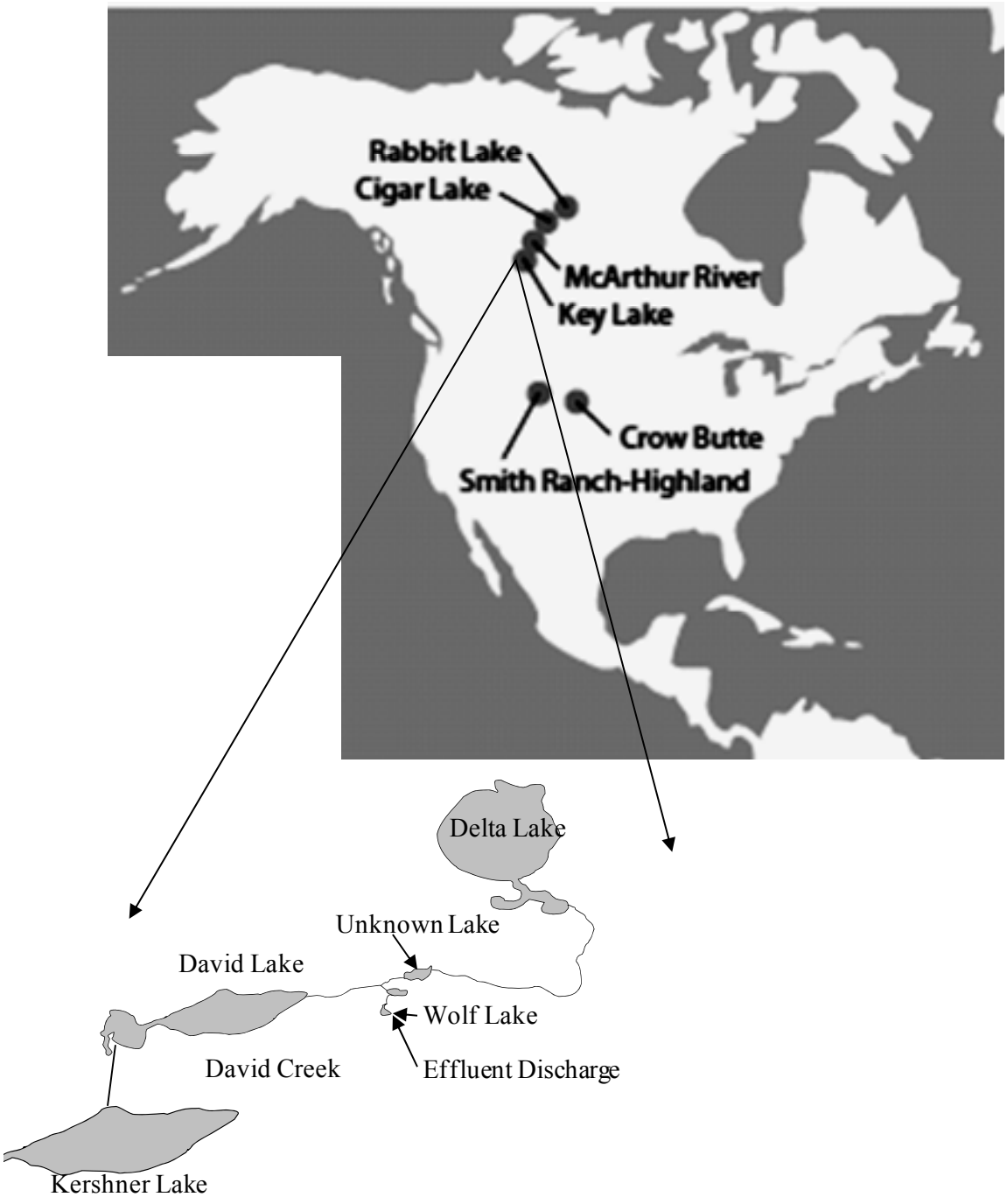

Figure 1.1 Map of North America indicating the location of Cameco Corporation uranium mining and milling sites. With inset detail of Key Lake facility. Figure modified from www.cameco.com and Driessnack et al. 2011a.

and is upstream of the discharge site. David Lake drains via David Creek which flows down to Unknown Lake. However, Kershner Lake has also been used as a reference site and is connected to David Lake. Wolf Lake drains into Fox Lake which drains via Yak Creek where it meets up with David Creek and drains into Unknown Lake, which is approximately 2 km downstream of the discharge site. Further downstream of Unknown Lake is Delta Lake, or about 10 km from the discharge site, with the system continuing on to the Wheeler River. David Lake has a surface area of 1.40 km^2, and mean depth of 2.44 m, Unknown Lake has a surface area of 0.14 km^2, and depth of 1.36 m and Delta Lake has a surface area of 2.85 km^2 and average depth of 4.42 m (Muscatello et al. 2008).

Prior to discharge into the receiving waters, the effluent is treated through a series of circuits that include bulk neutralization and reverse osmosis to reduce the level of contaminants. Treated effluent is initially released into monitoring ponds to be evaluated for pH, conductivity, TSS, ^{226}Ra, As, Ni, U, NH_3-N, Mo, and temperature. Only effluents with levels of these variables within permitted limits based on regulatory limits are released to the environment. If concentrations exceed limits, the effluent is recycled back into a reservoir for additional treatment prior to release (Cameco Annual Report 2006; 2007). In 2007, a Mo and Se removal process was added to the bulk neutralization circuit. This process acidifies the effluent to a pH of 3.5 causing Mo and Se to adsorb to ferric hydrite. This process is expected to reduce the annual average concentrations of Se to 0.01 mg/L and Mo to 0.3 mg/L in mill effluent based on both elements being identified as potential concern in EEM monitoring programs (Cameco Annual Report 2007). Success of this process will result in decreased effluent concentrations which will ideally result in lower levels of Se and Mo in the water column of the receiving environment. Se and Mo concentrations in the effluent currently average 0.017 mg/l (17.0 µg/L) for Se and 0.13 mg/L (130 µg/L) for Mo based on the 2010 annual averages compared to mean values in 2006 of 1.03 mg/L (1030 µg/L) for Mo and 0.029 mg/L (29.0 µg/L) for Se (Cameco Annual Report 2006; Anne Gent, Cameco Corporation, Saskatoon, SK, Canada, personal communication).

1.2 Selenium Background

Selenium (Se) is a metalloid and an essential trace element first identified by Swedish chemist Jöns Jacob Berzelius in 1817. Se is identified as element number 34 and is located in

group 16 of the periodic table with the chalcogens. Se displays similar physicochemical behaviours to sulphur which is also located in group 16 along with oxygen and tellurium (Wilber 1980; Suzuki 2005; Lenz and Lens 2009). Se exists naturally in the environment but exhibits an uneven distribution with some soils being Se-deficient while others are seleniferous (Riedel et al. 1991; Lenz and Lens, 2009). Various activities both natural and anthropogenic in origin can result in increased levels of Se in surrounding aquatic environments (e.g., agricultural run-off, soil leaching, metal mining, coal-ash activities) (US EPA 2004; Vidal et al. 2005). Also of interest is that Se has the ability to exist in different inorganic and organic forms or species in aquatic environments. Inorganic forms of Se are primarily selenate (+VI or SeO_4^{2-}), selenite (+IV or SeO_3^{2-}) and elemental Se (Se^0). Organic forms are selenides which can include selenoamino acids (e.g. selenomethionine), and methylated compounds (e.g. dimethyl selenide) (Bowie and Grieb 1991). Each of these species/forms have varying chemical, biological and toxicological properties (Andrahennadi et al. 2007; Miller et al. 2007; Lenz and Lens 2009).

Most commonly, the forms of Se entering an aquatic ecosystem are as selenate, selenite, or a combination of both. Se entering a system can remain free in the water column, can be absorbed or digested by organisms, or can bind or complex with particulates and/or sediment (Lemly 1999a). Additionally, depending on the environmental conditions, different transformations can occur. In oxidizing environments, selenite is slowly converted to selenate while in reducing conditions, such as in the porewater of anoxic sediments, selenate is reduced to selenite and then further reduced to elemental Se. Once Se is reduced to the elemental form it tends to precipitate in the sediment which extensively reduces its bioavailability (Bowie and Grieb 1991; Wiramanaden et al. 2010a). When evaluating systems exposed to Se, it is important to recognize the potential for Se to bioaccumulate. Due to a lack of biodegradation, Se has the ability to cycle between various compartments of the environment for many years (Lemly 2004; Muscatello et al. 2006). It has been reported that the first few centimetres of detritus and sediment may hold up to 90% of the Se in the system and due to the dynamic nature of these systems, can facilitate continued mobilization of Se into the food chain (Lemly 1999a). Therefore, it is important to address the sediment as it can function in the cycling of Se through the system when water concentrations are no longer elevated.

Mobilization of Se up the food chain begins at the level of the primary producers (algae, periphyton, and biofilm) where dissolved forms of Se (selenite and organic forms) are readily taken up (Bowie and Grieb 1991; Muscatello et al. 2008; Wiramanaden 2010a,b). Se is subsequently passed up the food chain to the primary consumers such as invertebrates (e.g. chironomids, mayflies). From here, small-bodied fish can consume the invertebrates as well as algae and biofilms. The small-bodied fish are then consumed by larger piscivorous fish (e.g. northern pike). Each step up in trophic level facilitates the transfer and biomagnification of Se to a point where higher trophic level fish populations may eventually be affected (Lemly 1997b).

As mentioned previously, Se is an essential micronutrient but has a very narrow range of deficiency and toxicity for fish (Hilton et al. 1980). With Se at 0.1-0.5 µg/g d.w. being required but levels > 3 µg/g d.w raising concerns of possible toxicity (Hodson and Hilton 1983). Se is required in the body for use in various enzymes, such as thyroid deiodinases, thioredoxin reductases and most notably glutathione peroxidase, a cellular antioxidant enzyme (Suzuki 2005; Miller et al. 2007). Toxicity from Se exposure is suggested to occur by two mechanisms. First, the improper substitution of Se such as in selenomethionine for methionine as an amino acid in proteins and can result in alterations in protein form and function (Orr et al. 2006). Se replaces sulphur (S) resulting in the formation of a triselenium linkage (Se-Se-Se) or a seleno-trisulfide linkage (S-Se-S), which prevents formation of the required disulfide (S-S) bond (Lemly 2002a). This erroneous substitution may be the result of a build up of a pool of selenomethionine in the body as this form is more stable then selenocysteine which is too highly reactive to store in the cell. The incorporation of selenocysteine into proteins is highly regulated by the genetic code which uses a specific codon (UGA) to incorporate selenocysteine into proteins. In contrast, selenomethionine is not specifically coded but competes with the normal amino acid methionine for incorporation into proteins (Suzuki 2005; Lu and Holmgren 2009).

More recently, another mode of toxicity has been identified as the generation of reactive oxygen species (ROS) specifically the superoxide anion $0_2^{\bullet-}$ resulting in oxidative damage, making Se a prooxidant instead of an antioxidant (Stewart et al. 1999). The free radical stress generated from Se toxicity is believed to cause cell death by apoptosis and/or necrosis. Studies have also noted different rates of apoptosis depending on the range and species of Se. Selenite has been noted to induce apoptosis while selenomethionine has not indicated induction potential

(Stewart et al. 1999). This induction of oxidative damage has received little attention in fish; however work by Misra et al. (2009; 2010) has begun to characterize the process in fish. This work has identified indirect evidence of a critical L-methionine-γ-lyase-like activity in fish similar to the process already identified in mammals. In mammals toxic levels of selenomethionine are detoxified by L-methionine-γ-lyase which metabolizes selenomethionine to CH_3SeH, α-ketobutyrate and ammonia. The CH_3SeH is then suggested to produce $0_2^{\bullet-}$ via GSH-mediated redox cycling (Chaudiere et al. 1992; Misra et al. 2009; 2010).

A unique feature of Se toxicity in fish is that adult populations often appear to be in satisfactory health while their progeny are affected due to the maternal transfer of Se deposited into eggs. The most noted effect of Se toxicity is increased incidences of larval deformities that tend to be skeletal in presentation (Schultz and Hermanutz 1990; Lemly 1997). The abnormalities are a result of uptake of excess Se from the yolk by the developing embryos. Characteristic deformities include scoliosis, lordosis, craniofacial and ocular malformations, and fin-fold abnormalities (Figure 1.2). In addition, edema has often been reported as a deformity although it may be reversible, and is not specific to Se toxicity (Lemly 1997a; Muscatello et al. 2006; Dr. P. Jones, University of Saskatchewan, Saskatoon, SK, Canada, personal communication). Increased incidence of deformities can eventually lead to the collapse of fish populations, as the mortality of deformed larvae is often greater than 80% which can severely limit recruitment into the adult population. Fish population collapses have been observed at Belews Lake, North Carolina USA, where exposure to elevated Se from a coal-fired electrical generating facility wastewater led to the eventual disappearance of multiple fish species from the system (Lemly 2002a).

Though the effect most commonly noted is larval deformities, toxic effects in adult fish have also been observed. Dilation of gill lamellae can be induced by Se and can contribute to impaired blood flow and gas exchange. Some hematological alterations have also been observed such as reduced hemoblasts and increased lymphocytes. There can also be impaired liver function as a result of structural changes from altered cell nuclei shape. Pathological changes in the kidney and heart which can inhibit proper function in adult fish have also been reported (Sorenson et al. 1984; Lemly 1993a; Lemly 2002a). Ovaries of exposed fish may contain swollen, necrotic or ruptured mature egg follicles. The eyes can also be affected in two ways,

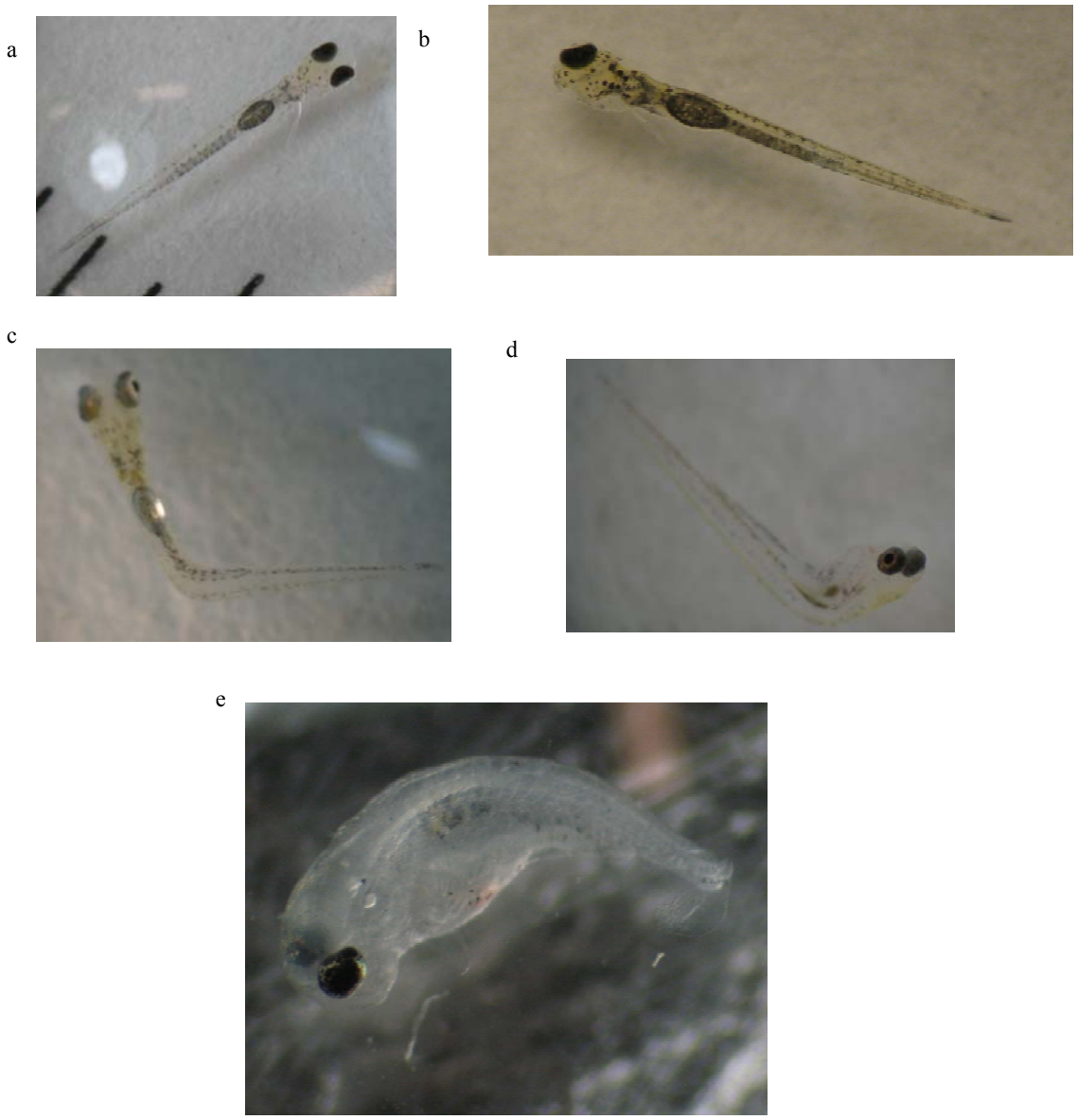

Figure 1.2 Normal fathead minnow (FHM) larvae (a), FHM larvae with an ocular deformity (b), FHM larvae with severe scoliosis (lateral curvature of the spine) (c), FHM with kyphosis (convex rounding of spine (d), FHM larvae with craniofacial abnormalities, body shortening, deformed yolk sac, and kyphosis

presence of cataracts or protruding eyeballs known as edema-induced exopthalmus (Sorenson et al. 1984; Lemly 1993a; Lemly 2002a).

1.2.1 Regulatory Guidelines for Selenium

The unique, dichotomous nature of Se being essential and toxic has made it challenging to determine appropriate guidelines and thresholds for environmental protection. A challenge also exists as to whether to establish individual guidelines for water, sediment, dietary or fish tissues or a combination thereof. It has been suggested that setting guidelines based on tissue residues is more suitable than water-based criteria and this idea is gaining acceptance (Reash et al. 2006). Also, Wayland and Crosley (2006) indicated that aquatic invertebrates can serve as excellent bioindicators of trace elements such as Se, as they are an integral trophic link. Setting guidelines based on invertebrate tissue residues which could also serve as dietary criteria for small-bodied fish also merits consideration for guideline development. Another possibility is setting guidelines based on sediment concentrations, as sediments provide habitat for invertebrates, the food source for small-bodied fish. A challenge exists here since sediment Se concentrations in a lake are variable and can vary depending on the amount of total organic carbon with increasing Se seen when TOC increases (Wiramanaden et al. 2010 a,b). However, all environmental matrices can be affected by the form of Se entering the system, the form in the sediment and the redox environment as well as the microbial community (Orr et al. 2006). Despite these challenges, the CCME has set a water guideline of 1 µg/L and the US EPA has proposed a water guideline of 5 µg/L and a whole body criterion of 7.91 mg/kg d.w. and 5.85 mg/kg d.w. for fish collected in late summer and fall. These values are based on the review of multiple studies evaluating the effects of Se (US EPA 2004). However, other proposed ranges and guidelines have been suggested such as whole body limits of 4 mg/kg d.w., female fish ovary limits of 10 mg/kg d.w. and a 3-4 mg/kg limit for the diet (invertebrates) (Lemly 1996; Wayland et al. 2006). An additional factor to consider is inter-species differences in sensitivities to elevated Se, such as differences in tolerance to Se between warm and cold water fish species (Chapman 2007; Miller et al. 2009). Therefore, to set a Se guideline extensive data on the amount and form of Se as well as physical site variables (e.g. sulphate, pH) need to be fully understood.

1.2.2 Selenium Speciation

The challenges surrounding Se have raised the need for information on the speciation of Se; an important consideration when establishing guidelines. Se speciation analysis can provide information that may allow more accurate and consistent predictions of Se uptake, distribution, transfer and bioaccumulation in an aquatic food chain (Wiramanaden et al. 2010 a,b). Recently there has been a growing body of work in the field of Se speciation and a significant amount of work has been initiated at the Key Lake facility (Wiramanaden et al. 2010a,b). Although total Se can be measured with standard methods (e.g., ICP-MS), these same methods cannot determine the speciation of Se. Methods now exist that have narrowed this gap; speciation can be determined using synchrotron based X-ray absorption spectroscopy (XAS) as well as ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS) and HPLC-ICP-MS where HPLC separates the Se species that are then detected by ICP-MS (Wallschläger and Roehl 2001; Wiramanaden et al. 2010b; P. Jones, University of Saskatchewan, Saskatoon, SK, Canada, personal communication).

1.3 Integrative Project Summary

A discussion of this work would be incomplete if the integrative goal of the overall "Key Lake Project" was not examined. The mesocosm work is one part of a much larger interdisciplinary project that has been ongoing at the University of Saskatchewan in partnership with Cameco Corporation for many years. Where extensive amounts of data have been collected and analyzed and are either published or in the publication process, much of which has identified Se as a causal agent for environmental impacts at the Key Lake facility. The data can be viewed in different phases, each of which builds on the previous: water/sediment, algae/biofilms and invertebrate (dietary exposure for fish), small-bodied and piscivorous fish. This has allowed the development of a uniquely detailed perspective on Se impacts on this aquatic ecosystem. The collected data will be discussed here in combination with information from the wider Se perspective to provide a detailed understanding of the impacts of Se as regulatory agencies move to better define regulatory guidelines for Se.

1.3.1 Water and Sediment

Work by Wiramanaden et al. (2010a) strove to identify the relationship between surface water, pore-water, sediment and invertebrate Se concentrations. These findings were then expanded upon with speciation work reported in Wiramanaden et al. (2010b). Those results found strong relationships between surface water, whole sediment and pore-water sediment Se levels. However, no clear relationship was identified between whole sediment and pore-water. The TOC content of the sediment was also evaluated and a relationship was identified between TOC and whole sediment Se concentrations on a lake by lake basis with TOC concentrations of greater than 10%. Particle size of the sediment was also considered and was weakly but negatively correlated with TOC. Also of note was the variability in Se levels observed in whole sediment samples within a site and within a lake. Variability was also identified in the pore water samples, where Se levels tended to be lower than surface water within a sampling site and lake. This was believed to be a reflection of the variability in the whole sediment Se concentrations. Again this further demonstrates the complex nature of Se not only between systems but within systems as well. The results of this work led the authors to conclude that Se in the water was not the only factor controlling the observed trends, but that the sediment had a role. This was why potential sediment effects were included in the mesocosm study. Se in the sediment and surface water were correlated yet this does not completely explain the movement of Se in this system, nor does it clearly demonstrate the role of the pore-water fraction and its redox environment (Wiramanaden et al. 2010a).

The speciation of Se in the lakes at the facility was presented in Wiramanaden et al. (2010b) where different proportions of Se species were noted between lakes. The form of Se in sediments from Unknown Lake was primarily red elemental Se whereas Fox Lake contained higher fractions of selenite and organic forms. It was also noted that high TOC sediment tended to contain primarily organic forms of Se compared to low TOC sediments where higher proportions of elemental Se was measured (Anton et al., unpublished data). Elemental Se is often viewed as being a less bioavailable form of Se than for example selenite (Wiramanaden et al. 2011b). When both the total Se and speciation results are considered, a picture of the movement of Se can begin to be constructed. Se enters the lakes primarily as selenate from the mill and then interacts with the sediment water interface and can be incorporated into pore-water. This pore-

water component appears to be key as the redox environment can begin to drive the transformation of selenate into different forms. A reducing environment favors the reduction of selenate to selenite and eventually elemental Se. In contrast, an oxidative environment favors the generation of selenate from selenite (Bowie and Grieb 1991). The redox environment can also influence the microbial community, where different species may accumulate higher levels of Se in different forms, such as the *Chlorophytes* that seem to accumulate low levels of Se (Schlekat et al. 2000; Baines and Fisher 2001). The redox environment can influence the forms and levels of Se in the primary producers and invertebrates that comprise the dietary components for small-bodied fish. Therefore, sediment composition and the redox environment appear to be combined drivers in the initial movement of Se into the upper trophic levels.

1.3.2 Primary Producers and Invertebrates

Uptake of Se into fish occurs primarily via the diet (Dobbs et al. 1996; Lemly and Skorupa 2007). It can therefore be assumed that primary producers and invertebrates are integral in the transfer of Se from the water/sediment into fish. The primary producer component comprised of algae, diatoms, microbes, and phytoplankton is a site for Se transformation along with the redox environment in pore-water. Again, the form of Se discharged from the mine is selenate. Work outside of Key Lake demonstrated uptake of selenate by algae appears to physiologically mediated where as selenite uptake is more rapid and most likely associated with selenite adsorbing to the cells rather than uptake into the cells (Riedel et al. 1991). The same author later showed transformation of selenate to selenite in algae and that selenate uptake increased as growth increased (Riedel et al. 1996). It has also been noted that the presence of sulfate can inhibit the uptake of selenate by algae, invertebrates and fish (Riedel et al. 1996; Simmons and Wallschläger 2005). Work by Muscatello et al. (2008) showed elevated levels of Se in the periphyton and plankton from impacted lakes at the Key Lake facility. Increased levels of Se in algae/biofilms were demonstrated in mesocosm work in 2007 and 2008 (Dubé and Harwood manuscript in preparation, Driessnack et al. 2011a). Speciation in the mesocosm work indicated Se in the forms of selenomethionine-like compounds, elemental Se, selenite, metal selenides, Se-GS, and DMSeO. This is interesting as selenocystine-like compounds were not identified in biofilm scrapings from the 25% effluent treatment (representative of Unknown Lake). Other speciation work determined that green algae exposed to selenate and selenite had

increased levels of selenomethionine after exposure (Umysova et al. 2009). The presence of DMSeO is important to note as work by Bowie and Grieb (1991) also saw evidence of the production of DMSeO and is produced by certain algal and microorganism species. DMSeO and other methylated selenides are volatile and outgassed from water, making this a possible removal route for Se in impacted systems (Bowie and Grieb 1991; Lemly 1997b; Simmons and Wallschläger 2005). It can be concluded that primary producers are accumulating Se at increased levels and in forms other than that discharged in the whole effluent in this Key Lake system, suggesting primary producers are part of the initial transformation into more organic forms of Se from the forms of selenate and selenite primarily from the water, pore water and sediment.

Moving up the food chain leads to an examination of invertebrate communities. Invertebrates are known to accumulate trace metals such as Se from water, sediment and diet thus consideration of their role is vital (Wayland et al. 2006). Muscatello et al. (2008) noted increases in Se levels in detritovores and predatory invertebrates. The high levels in the detritivores is expected as Orr et al. (2006) stated that transfer of Se through the food chain can occur via detrital pathways. In contrast filter feeding invertebrates had lower observed Se levels compared to the detritivores and predatory invertebrates (Muscatello et al. 2008). This is attributed to filter feeding invertebrates consuming primarily plankton while other invertebrates interact more closely to detritus and sediment. Interaction with sediment with higher TOC would likely result in greater Se exposure and potentially to higher proportions of organic forms of Se (Anton et al. unpublished data). The observed levels and forms of Se are most likely a function of the invertebrates feeding habitat, composition of their food, proportion of Se absorbed and whether the organisms gut microbes are capable of biotransforming Se. The biotransformation of Se may also be further influenced by unique digestive biochemistry (Andrahennadi et al. 2007). Se in the chironomoid, *Chironomus dilutus* has been examined in relation to the Key Lake site in various applications. Primarily in work by Franz et al. (2011) where it was determined in laboratory tests that *C. dilutus* do not take up Se as selenate as readily from the aqueous environment as selenite and selenomethionine. *In-situ* testing with *C. dilutus* led to the conclusion that accumulation of Se is via sediment not water exposure, although the relative and cumulative contributions of sediment, pore-water, detritus, and primary producers (algae/biofilm) are yet to be determined. This agreed with work by Wiramanaden et al. (2010a,b) that concluded a strong relationship between Se in chironomids and pore-water though

it may not necessarily be the primary route of exposure, where pore water may best represent the Se in sediment available to the chironomids. Speciation of Se in the invertebrates has demonstrated the presence of organic Se species, selenomethionine and selenocystine-like compounds, in whole-body *C. dilutus* (Wiramanaden et al. 2010b; Franz et al. 2011). More recently imaging of Se in chironomids has been done by Tse et al. (unpublished data) where Se was shown to be located across the entirety of the gut, the head and air sacs, suggesting that there may in fact be some aqueous Se uptake though to a lesser degree than via sediment and diet.

1.3.3 Small-bodied and Piscivorous Fish

The primary producers and invertebrates serve as a trophic link to the fish community, and at Key Lake this is primarily to the small bodied fish, lake chub (*Couesius plumbeus*) and spottail shiner (*Notropis hudsonius*) and the piscivorous northern pike (*Esox lucius*). In terms of Se it can be suggested that each step up in trophic level (water to primary producers to invertebrates to fish) may allow for more complete transformation and metabolism of Se species into organic forms (Andrahennadi et al. 2007). Extensive work has been done to evaluate not only the level and form of Se in fish, but also the potential reproductive and biochemical implications. Elevated levels of Se have been detected in fish downstream of the milling effluent discharge site as well as in mesocosm work, where some Se concentrations have been reported to be above the US EPA whole-body guideline of 7.91 mg/kg Se d.w. (Figure 4.1 and 4.2). Muscatello et al. (2006) evaluated the levels of Se in fish and eggs downstream of the discharge as well as the incidence of larval deformities. Increases in both deformities and Se levels were noted, with the deformities being primarily skeletal in nature as commonly associated with Se (Lemly 1993). Significant linear and quadratic relationships were characterized between type of deformities (edema, skeletal, finfold and craniofacial) and Se egg and muscle concentrations. Through this work using a weight of evidence approach Se was identified as a contaminant of primary concern in the David Creek drainage system at Key Lake.

Work by Phibbs et al. (2011 a,b) using lake chub and spottail shiners caged in reference and exposure lakes in contact with three different sediment types (sandy, medium organic content and high organic content) provided further data. Results indicated that whole body Se levels increased with increasing dietary exposure (organic content), and the fraction of selenomethionine like compounds was also noted to increase. Similar Se levels were noted

between feral small-bodied fish as with the non-native FHMs in the mesocosm systems, though FHMs have a wide distribution they have not been found in the David Creek Drainage system. These similar values and speciation profiles have led to the conclusion that the FHM is a useful reproductive analog for the small-bodied fish in these systems (Figure 4.2). The mesocosm work also determined increases in larval deformities, though increases were about 8-10% above that in reference water treatments compared to Muscatello et al. (2006) whose feral fish study saw 19-27% increase in deformities over reference. This difference has been attributed to a combination of the shorter exposure time for the FHMs used by Driessnack et al. (2011a) compared to the feral northern pike in Muscatello et al. (2006), as well as a possible species sensitivity difference.

Work has also evaluated the health status of fish in the exposed lakes, Kelly and Janz (2008) noted a significant positive correlation between Se in muscle and glutathione peroxidase activity. Yet evaluation of all results (i.e CF, HSI, weight) led to the conclusion that juvenile pike are experiencing limited oxidative stress in exposure lake sites. Earlier work has noted some decreases in CF in exposure lake fishes (Unknown (25% effluent) and Delta (5% effluent)), as well as changes in liver weight and LSI in males (Golder Assoc Ltd. 2005; Muscatello et al. 2006; Driessnack et al. 2011a). Fish swim performance was also assessed in 60 dph FHM larvae by Goertzen et al. (2011) using 5-day old larvae hathced from a separate laboratory based mesocosm study in the summer of 2009 by Driessnack et al. (manuscript in preparation). Subsets of 5-day post hatch larvae were collected and raised to 60 days in either 5% diluted milling effluent or reference, where larvae swim performance was then evaluated (Goertzen et al. 2011). Exposed larvae showed significantly decreased fork length, but overall analyses of weight-at-length showed significant increases in exposed larvae compared to controls. Evaluation of critical swim speed was significantly lower in exposed fish. In addition fatigued effluent exposed fish had reduced whole body triglycerides. The authors concluded that although effluent exposure negatively impacted swimming performance and altered metabolic status in laboratory reared offspring, the results are still somewhat unclear as to the biological significance and further investigation is warranted Goertzen et al. (2011).

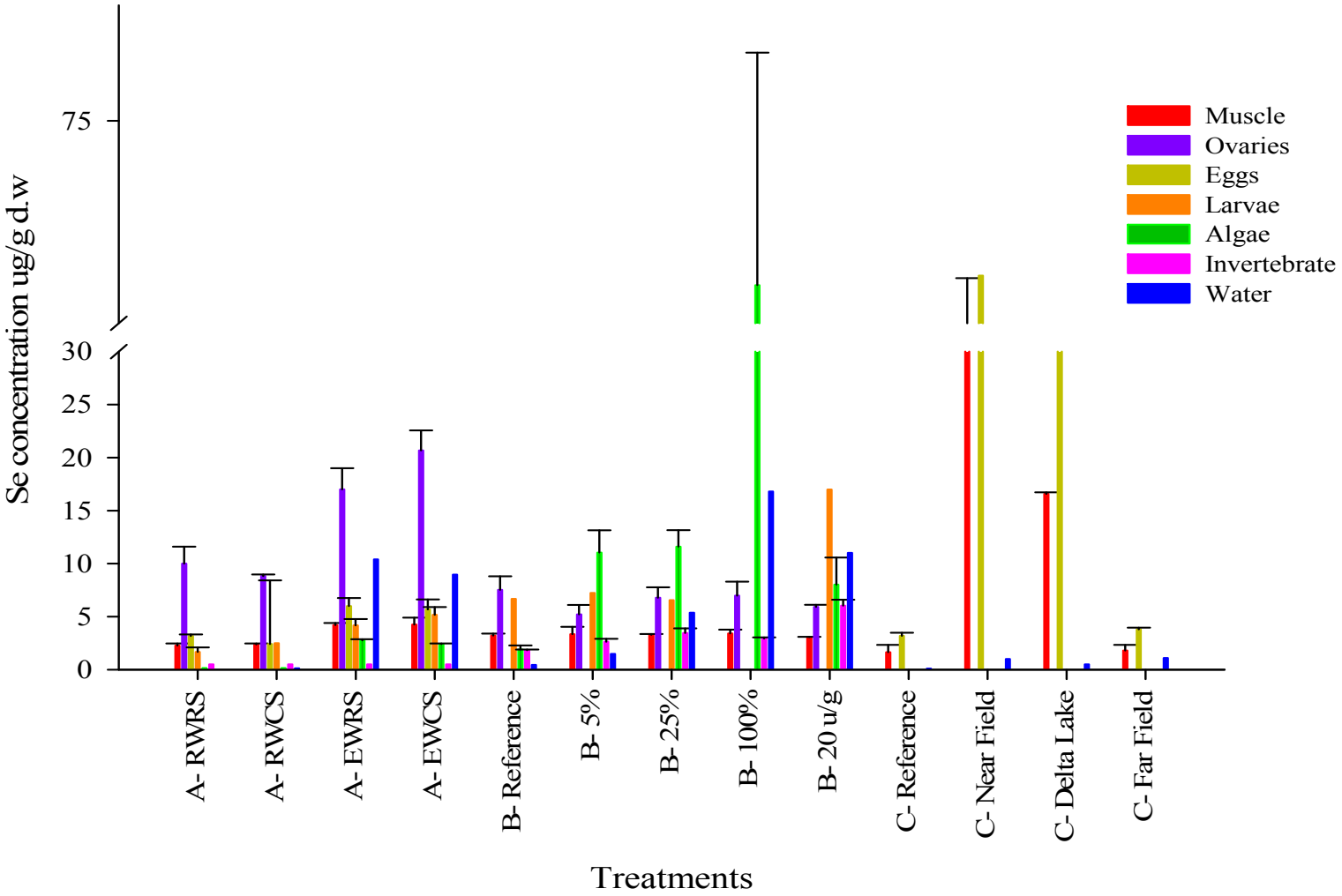

Figure 1.3: Concentration of selenium (Se) given in µg/g dry weight (d.w) for fish muscle, ovary, egg and larvae tissue from studies carried at the Key Lake facility as well as reported water, algae and invertebrate Se concentrations. Where for water µg/g should be considered µg/L. Reported values are from A- Driessnack et al. 2011a, B- Dubé and Harwood, manuscript in preparation, and C- Muscatello et al. 2006

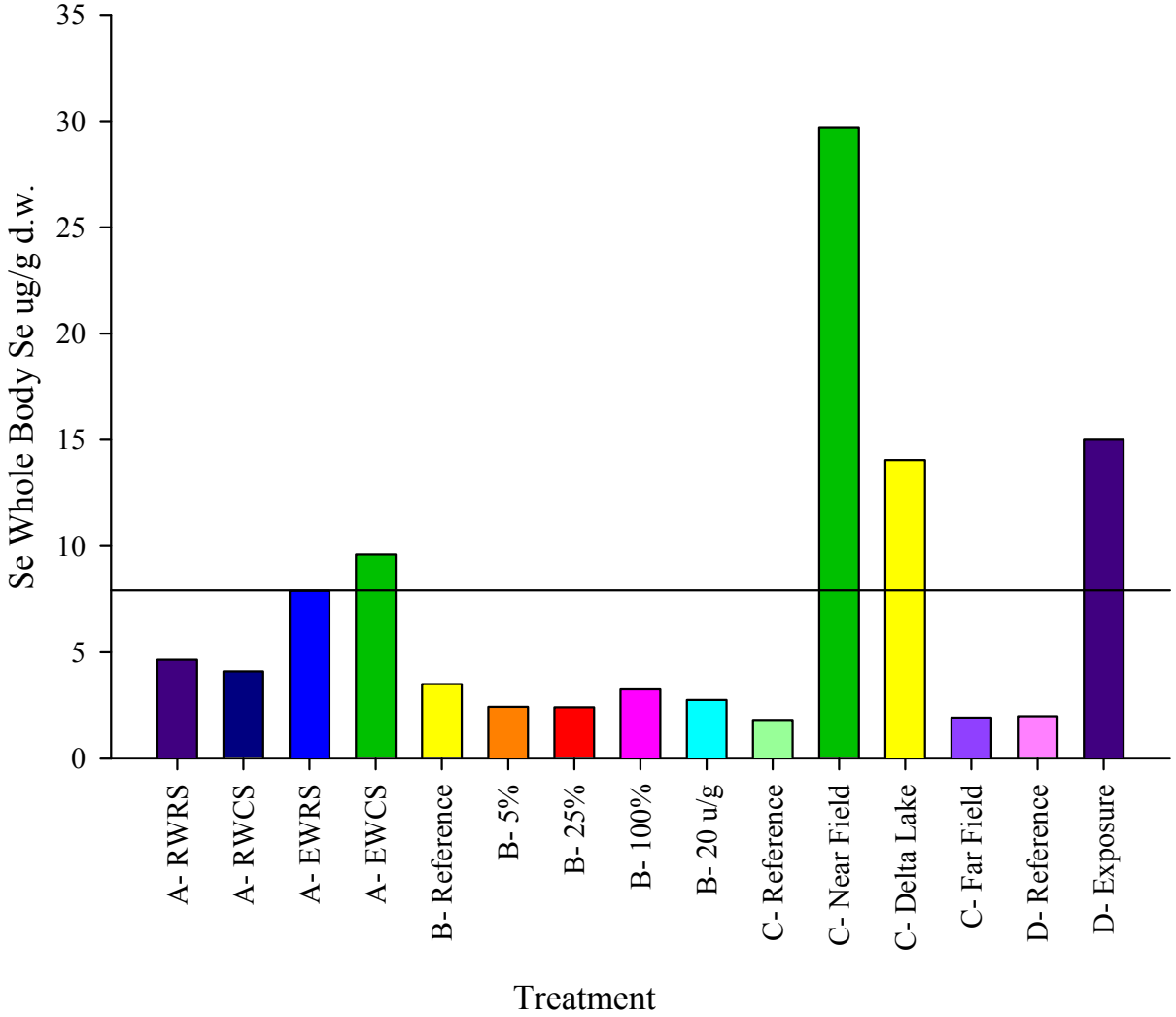

Figure 1.4: Whole body selenium (Se) concentrations (µg/g dry weight) in fish studies conducted at the Key Lake Uranium milling facility or using diluted effluent in mesocosms. Where the line represents the 7.91 mg/kg or µg/g d.w. guideline set by the United States Environmental Protection Agency, 2004 (Reported values are from A- Driessnack et al. 2011a, B- Dubé and Harwood, manuscript in preparation, C- Muscatello et al. 2006, D- Phibbs et al. 2011a

The results of the fish work are interesting as through Muscatello et al. (2006, 2008), Driessnack et al. (2011a) and Phibbs et al. (2011 a,b) three native species of fish (northern pike, lake chub, spottail shiner) and one non-native, fathead minnow, accumulate Se following exposure to the milling effluent. Through Muscatello et al. (2006) and Driessnack et al. (2011a) it has been identified that increases in larval deformities are evident following effluent exposure, and these indicate alterations in reproductive ability in fish. In regards to the speciation results and how they provide evidence of the movement of Se, Phibbs et al. (2011a,b) and Driessnack et al. (2011a) showed increased levels of selenomethionine like compounds in fish whole body, muscle, ovary, eggs and larvae following exposure. Field collected invertebrates, which the caged fish would have ingested, showed greater proportions of selenomethionine like compounds compared to reference with this trend translating to the small-bodied fish. This has helped visualize the movement of Se into the fish from the system. This was also seen with the mesocosm work where FHMs showed increased selenomethionine-like compounds following exposure. It is interesting that the exposure was through the water and sediment and the fish were fed a controlled diet of frozen bloodworms. Se speciation of the control bloodworm diet showed selenocystine like compounds suggesting the source of selenomethionine like compounds was from the algae/biofilms. Also to consider is that it may be that the fish breakdown the selenocystine and scavenging of Se into Se-methionine. Both studies indicated the importance of both the invertebrate and primary producer exposure routes to fish.

From this data a generalized sequence of events for the bioaccumulation of Se at the Key Lake facility can be proposed (Figure 4.3):

1. Se enters the system in the form of selenate at Wolf Lake
2. Transformation begins in the immediate downstream lake systems (Wolf and Fox), although most of the research of late has focused on the lakes further downstream (Unknown and Delta). This is seen with the presence of different Se species in the sediment and pore water in the work by Wiramanaden et al. (2011a,b) and in the mesocosm work by Driessnack et al. (2011a) saw transformation in the artificial streams. There are interactions occurring at the sediment-water interface, as the pore water begins the reduction of selenate to selenite and eventually elemental Se. This has been noted in

the sediment with a predominance of selenite at Fox Lake sites and elemental Se at Unknown Lake.

3. The primary producer component consisting of biofilms and algae take up Se in the form of selenate as well as potential adsorption of selenite. The various species of organism have different uptake rates and levels to which they concentration Se as well as different metabolic processes. This is where as in the mesocosms the great speciation variety may be a reflection of the diverse taxonomy of primary producers. An important result of note from the mesocosm work was the presence of DMSeO, a form of Se that represents a potential route for Se to be volatilized from the system (Bowie and Grieb 1991; Lemly 1997b).
4. From here the primary producers are consumed by the invertebrate communities. Again species differences will influence uptake. Muscatello et al. (2008) noted higher levels of Se in detritivorous invertebrates compared to filter feeders, which coincides nicely with the association noted by Wiramanaden et al. (2010a) that sediments with higher TOC tend to have higher Se levels. These higher TOC niches would be more suited to the detritus consuming invertebrates. With the speciation work we see that initial shift to predominance towards selenomethionine-like compounds occur following exposure to increased Se from the effluent. This can be related to biochemical scavenging processes which collect Se and sequester it in the Se-methionine pool (Suzuki 2005; Driessnack et al. 2011a)
5. The now predominately organic selenium forms are translated to the fish, the small-bodied fish first and then the piscivores. Though Muscatello et al. (2008) noted increased levels of Se in exposed small-bodied fish and northern pike there was no evidence of biomagnification between the two fish groups.
6. Fish are more sensitive to increased Se than the primary producer and invertebrate communities, so the increased Se in their tissue has often led to reproductive implications. Most commonly noted are increased incidences of deformities in offspring which can lead to eventual collapse of fish populations due to a lack of recruitment of healthy juveniles to eventually replace the adults (Lemly 1997a; Muscatello et al. 2006). In the body Se tends to be stored in pools of selenomethionine as this form is less reactive then selenocysteine. Yet pools of selenomethionine can greatly increase the chance of

When examining effects on various freshwater fish species and their affected habitats it is evident when dealing with Se that there are very strong interspecies variations to be considered. Not only is the concentration of Se from the input sources important, but what form or species of Se is present and the species of primary producers present and the redox environment. A reducing environment will allow for elemental Se to be a product that can result in the sediment serving as a Se reservoir, whereas that would not be as large a component in an oxidizing environment. Also the invertebrate community, if there is a larger portion of detritovores then a large fraction of Se may be available for transfer into fish. Another factor to consider with the invertebrate communities is potential nutrient enrichment from industrial discharges where nitrogen and phosphorous have been reported with pulp mill studies (Culp et al. 2000; Cash et al. 2003). These factors have also been investigated at Key Lake (Kelly and Janz 2008). Shifts in benthic communities have been reported in lakes downstream of uranium mining and milling sites (Robertson EL 2006, Master Thesis, University of Saskatchewan, Saskatoon, SL, Canada; http://library2.usask.ca/theses/avialable/etd-12212006-21225/unrestricted/ErinRobertsonThesis.pdf), and the dominant invertebrate species should be considered, since certain species may accumulate higher levels of Se than less prevalent

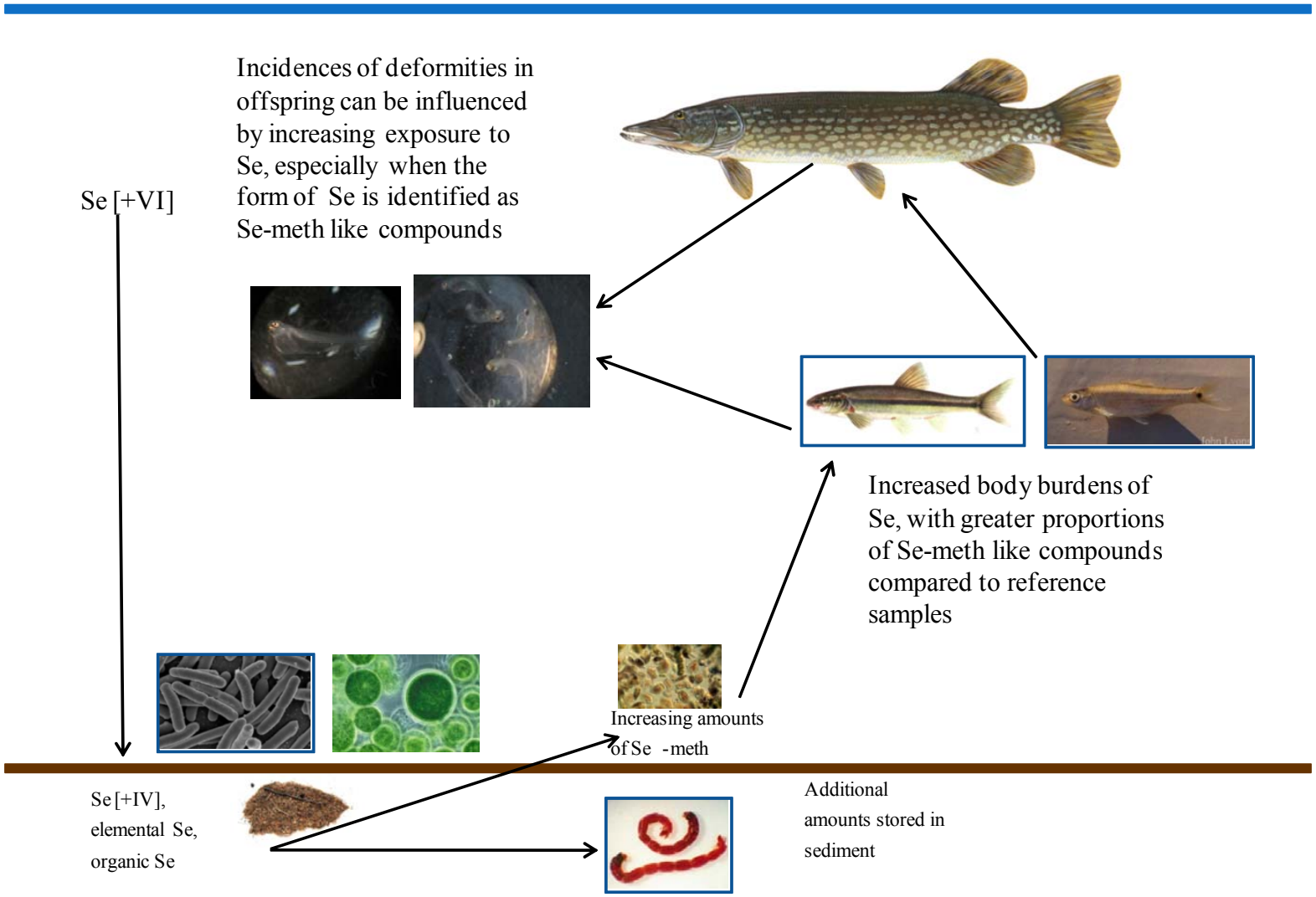

Figure 1.5: Conceptual model of the movement and speciation of selenium (Se) through the Key Lake facility. Where Se [+VI] indicates selenate, Se [+IV] is selenite, Se-meth is selenomethionine like compounds.

invertebrate species. These potential increases and changes in the dietary exposure pathways for fish need to be understood as they serve as an important trophic link between water and fish. Then there are the fish species to consider; some such as the mosquitofish are more tolerant than others (Lemly 2002). Fathead minnows may also not be as sensitive as a function of their digestive biochemistry where uptake of selenate would be favored over selenite, which is suggested to be more likely to bioaccumulate in nature than selenate (Ogle and Knight 1989).

1.4 Metal Mining Effluents

Although the effects of Se have been of primary interest at the Key Lake facility it must be noted that the discharged effluent is complex and also contains elevated levels of metals such as Na, As, Co, Cu, Pb, Mo, Ni, U, Zn, ^{210}Pb, ^{210} Po, ^{226}Ra and ^{230}Th as well as chloride, ammonia, and sulphate (Klaverkamp et al. 2002). Metals may also not always be the only elevated constituents, other compounds used during extraction such as hydrocarbons, polymers and alcohols for instance could potentially be present and contributing or interacting with metal toxicity. Domestic sewage is also discharged with the effluent creating the possibility for the presence of endocrine disrupting chemicals. The discharged effluent has also contributed to changing the receiving environment downstream of the discharge site at Wolf Lake from a soft water to hard water system (CNSC 2006; Muscatello et al. 2006; Muscatello et al. 2008; Rozon-Ramilo et al. 2011). Therefore, complex industrial effluents make it challenging to assess cause and effect linkages for single contaminants when there are various synergistic and antagonistic relationships between the various components (e.g., Se and Hg or As) and where the toxicity of a contaminant can be affected by the water chemistry (e.g., hardness, sulphate, pH) in the receiving environment (Pyle et al. 2002a; Dubé et al. 2005; Simmons and Wallschläger 2005). A large number of studies exist that have examined the implications of industrial effluent exposure on fish ranging from behavioural, biochemical, hormonal, to reproductive endpoints (Dubé et al. 2005). Thus far, some of the effects identified include changes in liver size, egg size, reductions in growth and condition factor and increased metal tissue burdens in multiple fish species (Levesque et al. 2002; Weber et al. 2008). In fact, work carried out at Key Lake reported mortalities of 70-90% in fathead minnow larvae caged in an exposure lake downstream of the discharge site (Pyle et al. 2001). Rickwood et al. (2006 a,b,c; 2008) have shown repeatedly that fathead minnows exposed to industrial effluents (i.e., pulp mill, metal mining effluent) have

altered egg production and reduced offspring survival, increased larval deformities and increased metal body burdens.

1.5 Fathead Minnow

The fathead minnow (FHM) (*Pimephales promelas*) is a small-bodied fish of the Cyprinidae family, one of the largest families of freshwater fish. The species occupies a wide range of habitats in North America which has resulted in this species becoming important in ecotoxicological studies (Benoit et al. 1982; Ankley et al. 2001). Fathead minnows have been used extensively in laboratory and field-based studies providing a database on their lifecycle and successful culturing methods. Various government regulatory agencies use the FHM in regulatory and monitoring work such as the United States Environmental Protection Agency (US EPA), Organization of Economic Cooperation and Development (OECD) and the Canadian Environmental Effects Monitoring (EEM) Program (Munkittrick et al. 2002; Ankley and Villeneuve 2006). FHM are an ideal test species as they are small with mature adults measuring 50-75 mm long, mature males weighing 4-5 g and females 2-3 g. The mature males and females are relatively easy to identify as they are sexually dimorphic. Males exhibit nuptial tubercles, and a fleshy pad from the nape to the dorsal fin that has been referred to as a fatpad. Females can be identified by the presence of a fleshy ovipositor during reproduction (Parrott 2005; Ankley and Villeneuve 2006). Finally, FHM are tolerant to a wide array of water quality variables such as pH, temperature, alkalinity and hardness (Ankley and Villeneuve 2006). When optimum conditions are provided FHM can easily produce 50-150 eggs per spawning event at three to four day intervals. The fertile eggs remain clear for 36-48 hrs and then transition into the eyed stage. The eggs typically hatch in four to five days also making it easy for larval endpoints to be examined (Ankley et al. 2001; Jensen et al. 2001).

1.6 Artificial Streams

Modular mesocosm artificial stream systems have been utilized for over a decade to evaluate and assess the effects of single contaminants and complex mixtures on algae, invertebrates and fish (Dubé et al. 2002 a,b; Cash et al. 2003; Hruska and Dubé 2004; Rickwood et al. 2006 a,b; 2008). Artificial stream systems are a valuable tool in the integration of laboratory and field studies (Figure 1.3). They provide the environmental relevance of field studies while maintaining the control of multiple variables as in laboratory studies. Where the

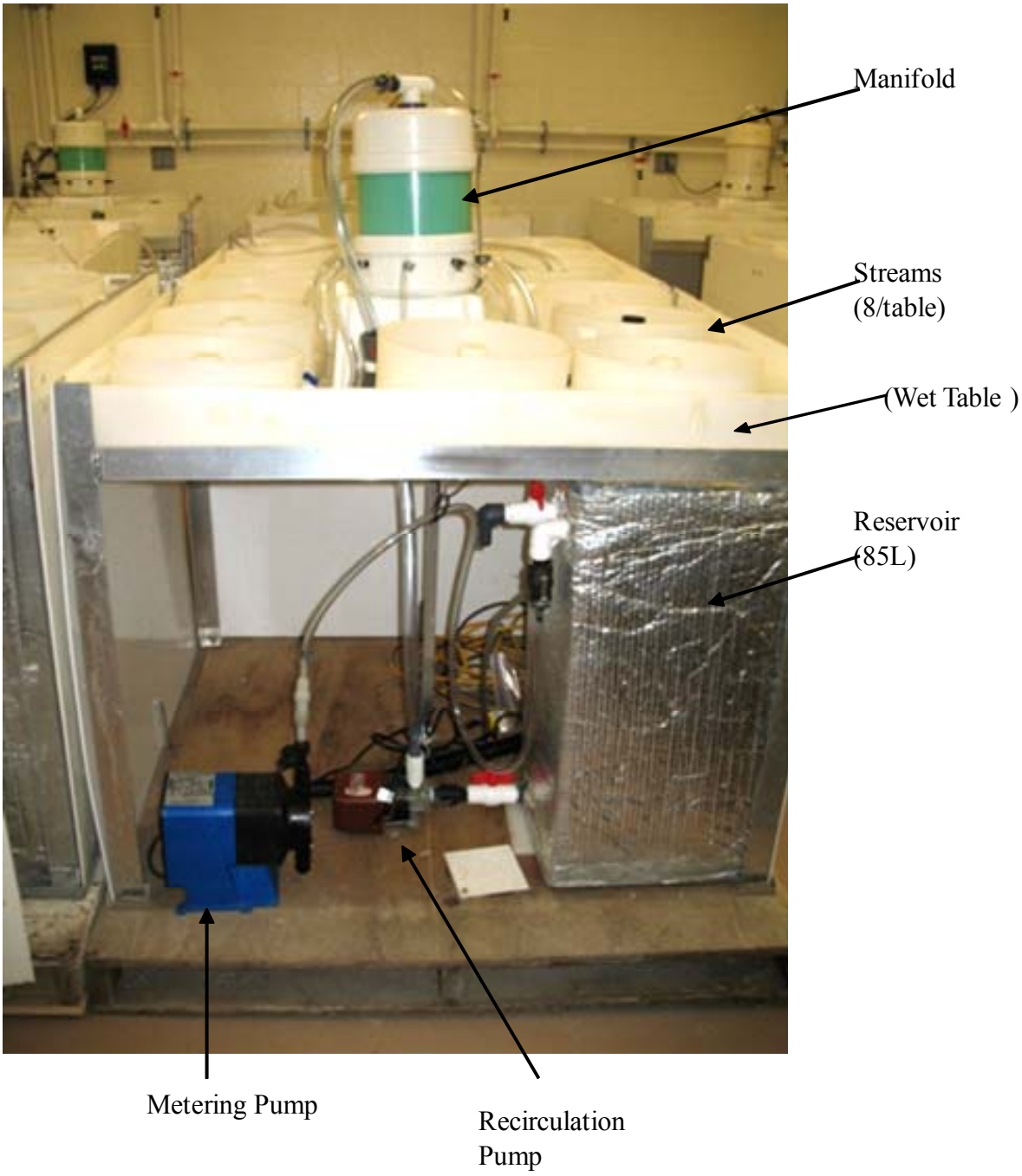

Figure 1.6: Schematic of the modular mesocosm systems used in the 21-day fathead minnow partial lifecycle reproductive bioassay. The system dimensions are 122cm x 122cm x 107cm (length x width x height).

systems can be set-up in the field where water, sediment and primary producer communities from the study sites can be included in the systems. They allow for control of exposure and exposure routes, experimental duration, temperature and water quality with the added benefit of increased statistical power for complex hypothesis testing compared to field studies (Culp et al. 2003; Dubé et al. 2002a). The mesocosms work by pumping water from a reservoir, located below a wet table, using a recirculation pump which moves the water into a pressurized manifold. The manifold then distributes the water to each of the 8 streams on the wet table. Water is then allowed to flow through the streams and drain back into the reservoir, where a metering pump controls the number of turnovers per day. The mesocosms used here may be better described as microcosms due to their size. However, to maintain consistency with the use of these tables in the literature they will be referred to as mesocosms. Additionally, mesocosm stream systems are an accepted method for Canada's Environmental Effects Monitoring (EEM) programs due to the complications in collecting sufficient amounts and of high quality data from some field monitoring situations (Munkittrick et al. 2002).

EEM is a cyclical monitoring program required by all Canadian mines under the Metal Mining Effluent Regulation (MMER) through the *Fisheries Act* to assess the effects of metal mining effluents on fish habitat, fish and the use of fisheries resources. EEM monitoring is grouped into phases: 1) initial monitoring, 2) periodic monitoring (2-6 year cycles), 3) focused monitoring, and 4) investigation of cause (IOC). During the monitoring, interest is placed on three main areas: 1) fish health through surveys, 2) benthic invertebrate surveys, and 3) mercury levels in fish. EEM response endpoints for fish were included in this thesis research to allow for comparison with other fish work in Canada both at the Key Lake facility as well as other mine sites. The Key Lake facility has recently moved into the IOC phase of monitoring (Lowell et al. 2007; www.ec.gc.ca/eem, M. Dubé, University of Saskatchewan, Saskatoon SK, personal communication).

1.7 Relevant Endpoints

Studies evaluating effects of Se need to address endpoints that represent the health and status of the offspring due to the maternal transfer of Se. Recommended endpoints include egg size, fertilization and hatching success of eggs, larval survival or mortality, and incidences of

larval deformities. These recommendations are based on endpoints that are required in EEM and endpoints that have been evaluated in prior FHM mesocosm studies (www.ec.gc.ca/eem; Rickwood et al. 2006 a,b,c,; 2008; Rozon-Ramilo et al. 2011). The reproductive ability of the adults should be considered in terms of the number of spawning events, cumulative spawning events, cumulative egg production, cumulative eggs/female/day, mean eggs, mean eggs/female/day and mean total eggs/female/day. The health of the adults can also be evaluated by measuring and calculating body weight, fork length, liver and gonad weight, condition factor (CF), gonadosomatic index (GSI) and liversomatic index (LSI). Many of these endpoints (GSI, LSI, CF, survival) are also required in EEM monitoring programs as provided in the Technical Guidance Documents for this program (www.ec.gc.ca/eem). Various samples of media and tissue should be collected for metals analysis. Examples of samples include water, sediment, biofilm/algae, invertebrates, fish muscle, fish gonad (especially female ovary for Se work), eggs and larvae. These types of samples can also be evaluated for their Se speciation so that information on the transformation and transfer of Se through a system can be evaluated and related to reproductive endpoints.

1.7 Research Objectives

The overall objective of this field-based mesocosm project was to evaluate the effects of uranium milling effluent elevated in Se compared to reference lakes on the reproduction of FHMs and implications for the health of the adults and offspring. The study was also designed to simultaneously evaluate the water and sediment exposure pathways in a two factor design. In addition, this thesis provided the opportunity for relational analysis of results between Se tissue burdens and responses for multiple tissues and responses.

Objective 1: To conduct a field-based mesocosm bioassay using FHMs to evaluate the relative and cumulative contributions of water (effluent) and sediment exposure on survival, reproduction and tissue metal burdens.

Ho: Exposure of adult FHMs to 25% treated uranium mill effluent has no significant effect on the reproduction and health of FHM adults or offspring in the presence or absence of historical sediment contamination.

***Objective 2*:** To determine the contribution of Se (based on total Se analysis and Se speciation) in sediment and water to any effects observed and to deduce which route of exposure (water versus sediment) is having a greater effect on fish.

Ho: The total concentration of Se and the proportions of its forms (speciation) will not differ in fish tissues based upon exposure via water, sediment or both routes

***Objective 3*:** To examine in detail the relationships between Se tissue burdens and fathead minnow responses for multiple tissues and responses (adult and offspring) using the relevant and controlled exposure environment of an artificial stream system.

Ho: The relationships between low and high concentrations of Se in different matrices (environmental and fish tissue) and reproductive endpoints will not differ.

CHAPTER 2:

The use of field-based mesocosm systems to assess the effects of uranium milling effluent on fathead minnow (*Pimephales promelas)* reproduction

Driessnack MK, Dubé MG, Rozon-Ramilo LD, Jones PD, Wiramanaden CIE, Pickering IJ (2011). The use of field-based mesocosm systems to assess the effects of uranium milling effluent on fathead minnow (*Pimephales promelas)* reproduction. Ecotoxicology (2011) 20: 1209-1224.

2.1 Introduction

Anthropogenic activities related to irrigation and mining for resources such as uranium have led to an increase in metals in surrounding aquatic environments (Vidal et al. 2005; Chapman 2007). Increases of metals and metalloids in the environment are a potential threat to the survival of organisms inhabiting the impacted area (Bervoets et al. 2005). Selenium (Se) was recognized as an important environmental contaminant in the 1970's and 80's (Swift 2002) and its toxicity to fish from industrial activities in North America, Australia and New Zealand (SETAC Pellston Workshop 2009) has lead to an increase in the number of studies examining concentrations of Se in fish, sediment, and water at locations downstream of milling and mine operations (Pyle et al. 2001; Klaverkamp et al. 2002; Muscatello et al. 2006; Wiramanaden et al. 2010a).

Interest in Se arises from the element being essential but also toxic to fish within a small concentration range (Dobbs et al. 1996). Se plays a role in the regulation of redox intracellular signaling and homeostasis and thyroid hormone metabolism in animals (Suzuki 2005). The amino acid selenocysteine is a component of proteins that protect against free radicals such as the cellular antioxidant enzyme, glutathione peroxidase (Lemly 1997a; Orr et al. 2006). Glutathione peroxidase reduces hydrogen peroxide to water and organic hydroperoxides to more stable derivatives, thus protecting the cell membranes and other cellular contents from damage (Bell et al. 1987; Lemly 1997a). Se toxicity may result from the replacement of sulfur with Se in other amino acids and the subsequent incorporation of these selenoamino acids into proteins. This could result in modification of the three-dimensional structure of the proteins thereby altering their function (Orr et al. 2006). Excess Se can also have teratogenic effects on developing fish. The high level of Se in the female is deposited into the yolk of the developing eggs and as the embryo develops it uses that yolk as an energy source resulting in increased exposure to Se (Muscatello et al. 2006).

When Se enters an aquatic system three things can occur; it can be absorbed or digested by organisms; bind or complex with particulate matter and surface sediments; or can remain free in the water. Each of these occurrences allows for Se to bioaccumulate in aquatic systems (Vidal et al. 2005; Muscatello et al. 2006; Lemly 1999a). Unlike organic contaminants, Se does not biodegrade and therefore has the ability to move and cycle between different parts of the

environment where it can persist for years (Lemly 2004). Se accumulation in sediment is very important when looking at long-term exposure as dynamic mechanisms can make Se available and mobilized into the food chain for long periods of time. Accumulated Se is typically limited to the first few centimeters of the sediment and overlying detritus, with estimates of up to 90% of the total Se in the system located in sediment (Lemly 1999a).

Se can exist as four common oxidation states and multiple species each varying in its chemical, biological and toxicological properties (Miller et al. 2007; Andrahennadi et al. 2007; Lenz and Lens 2009; Dobbs et al. 1996). The four forms include organic selenides, elemental Se, selenite, and selenate, with all but elemental Se capable of existing in a dissolved form (Bowie et al. 1996). Forms commonly found in the environment include selenite (Se^{+IV}) and organic selenides which are highly mobile and very bioavailable, while elemental Se and selenate (Se^{+VI}) are the less bioavailable forms; selenate and selenite are the form typically entering the aquatic environment (Lemly 1999a; Fournier et al. 2005; Andrahennadi et al. 2007). Included within the organic selenides are selenomethionine and selenocysteine. Limited work has been done on the speciation of Se in sediment, water, algae, invertebrates and fish, due to the difficulties inherent in measuring speciation in these complex samples.

Examination of Se effects on fish populations are facilitated by approaches and methods targeting reproductive processes under controlled exposure conditions (Thorpe et al. 2007). The fathead minnow (*Pimephales promelas)* is ideal for reproductive bioassay assessments as they are small, can be successfully grown and bred in the laboratory and are sexually dimorphic (Parrott 2005). They are also tolerant of a wide range of water quality variables such as pH, temperature, alkalinity and hardness (Ankley and Villeneuve 2006). The partial lifecycle reproductive assay using fathead minnows as outlined by Ankley (2001) and modified by Rickwood et al. (2006a,b; 2008) is a useful tool in assessing the effects on both adult fish and their offspring.

Previous work has used fathead minnows (FHMs) to evaluate the effects of various mine effluents (Pyle et al. 2001; Rickwood et al. 2006a,b; 2008) as well as many studies which have used mesocosm or artificial stream systems for experimental exposure under environmentally relevant conditions (Dubé et al. 2002b; 2005; 2006). Work by Pyle et al. (2001) reported 70-90% mortality occurred in larval FHMs that were exposed *in situ* to treated mine effluent from a

uranium mine in northern Saskatchewan, Canada. While the work by Rickwood et al. (2006a) with FHMs in mesocosms focused on pulp and paper effluent it provided a model for experimental designs in terms of larval deformities and endpoints. In other work with multi-trophic mesocosm systems and FHMs, Rickwood et al. (2008) showed that exposure to metal mine effluent increased egg production and spawning events possibly leading to inferior eggs. Rickwood et al. (2006b; 2008) conducted multi-trophic and water only exposures and while they reported increased reproduction in trophic transfer streams, reduced reproductive output was evident only in the exposure through water treatments and not through the combination of water and the diet (multi-trophic). This emphasized the need to better understand the pathways of fish exposure to metals, especially Se and the effects that are mediated through water, diet and sediment exposures.

In this study, the FHM partial lifecycle reproductive bioassay as modified by Rickwood et al. (2006a,b; 2008) was used to assess the effects of mine effluent, in the presence and absence of sediment contamination, on reproduction and survival of the adults and larvae. The study was designed to separate water exposure representing current effluent conditions from sediment exposure representing historical contamination. Understanding the source of exposure is essential to inform regulatory practice and to direct and assess potential mitigation scenarios. Total metal and Se speciation analysis of water, sediment, algae and tissue were used to understand how Se affects aquatic compartments. Potential changes due to Se were evaluated in the context of the effluent mixture. Mesocosm systems were used to provide an environment for statistically replicated experimentation where exposure to mine effluent could be controlled and pathways of exposure (effluent and sediment) evaluated separately and in combination. This study is also one component of a large interdisciplinary project to evaluate the impacts of Se at the Key Lake site (e.g. Wiramanaden et al. 2010a,b).

2.2 Materials and Methods

This study was conducted in June and July of 2008 at the Key Lake uranium milling facility in north-central Saskatchewan, Canada (57°11′N, 105°34′W). The site is located approximately 600 km north of Saskatoon, SK (Figure 2.1). At the facility, uranium ore is milled and the resulting treated mill effluent is discharged into the aquatic environment leading to

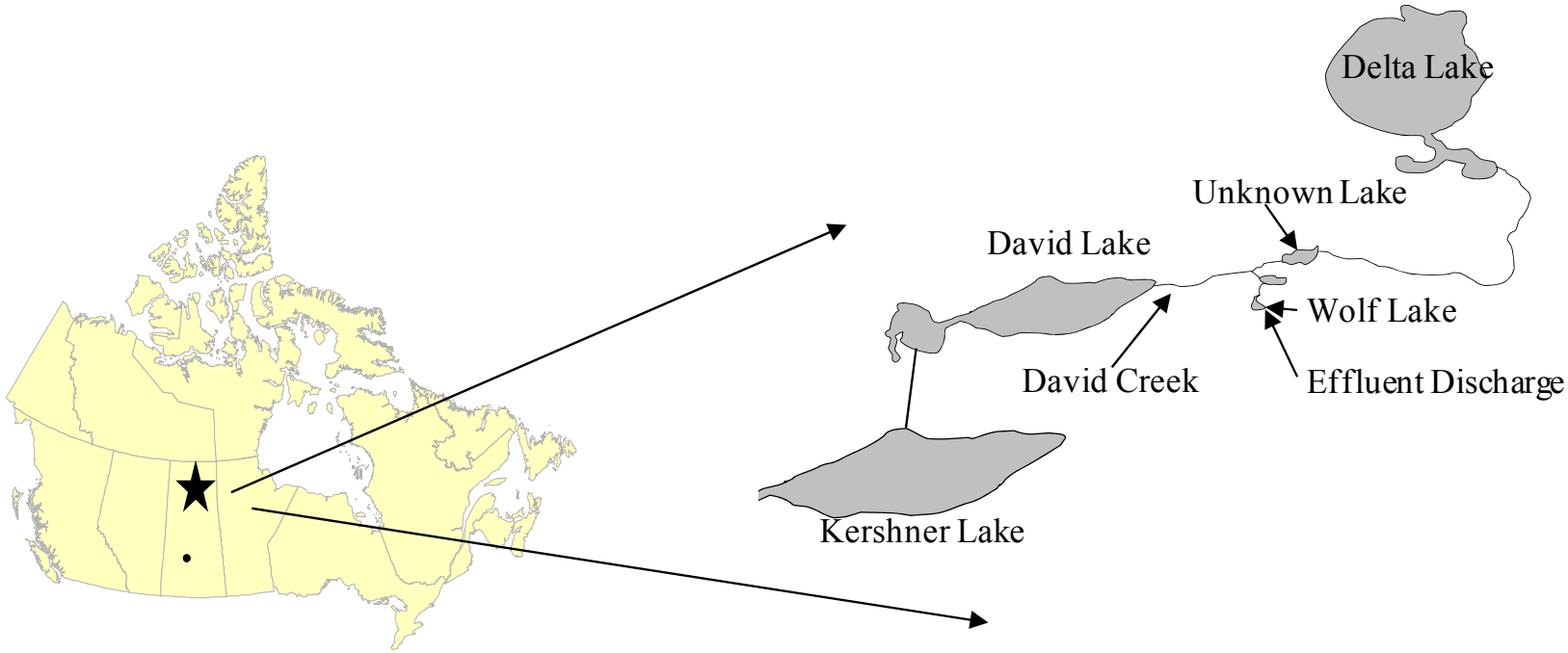

Figure 2.1: Map of Canada indicating the location of the Key Lake milling facility in Northern Saskatchewan

increases of metal concentrations, including Se in downstream environments (CNSC 2006). The milling facility discharges treated effluent into the aquatic environment at Wolf Lake. In 2007, 1,552,868 m^3 of treated milling effluent was discharged into Wolf Lake, which is part of the David Creek drainage system. The effluent that is discharged to the receiving environment contains Na, Co, Ni, Cu, Zn, As, Se, Mo, Pb, ^{210}Pb, ^{210}Po, ^{226}Ra, ^{230}Th and U in addition to chloride, ammonia, and sulphate.

The sediments and water used in the study were collected directly from the lakes in the David Creek drainage. The sediments collected were from areas near the edge of lakes and was primarily sand of low organic content. Higher organic content sediments could not be used due clogging and blocking of the pumps in the mesocosm systems. In each stream three cups of sediment were placed in the bottom, to give a sediment depth of 3.8 cm (1.5in). The experimental exposure was conducted for 21-days using field based mesocosm systems. Each mesocosm (one treatment per mesocosm) consisted of eight replicate, 10.3 L circular polyethylene streams. The streams rested upon a wet table that drained into an 85-L reservoir from where it was pumped back to the mesocosm streams. Turnover of exposure solutions was performed twice daily by use of a metering pump connecting the larger mixing head tanks to each mesocosm reservoir. These systems have been described in more detail by Hruska and Dubé (2004).

2.2.1 Pre-exposure Design

Trios of six-month old naïve FHMs (fathead minnows) (1 male, 2 females), in that they have not been allowed to reproduce prior to being introduced into the mesocosm streams, purchased from Osage Catfisheries, Missouri, USA. Fish were randomly selected from stocks of FHMs maintained at the University of Saskatchewan until used for research. Fork length (cm) and weight (g) were measured and secondary sex characteristics assessed. Secondary sex characteristics of males were examined as the presence or absence of banding, nuptial tubercles and fin dot in addition to a 0-4 ranking of the dorsal fatpad. Females were examined for ovipositor size and given a ranking of 0-4. Fish were then randomly placed into mesocosm streams with a spawning tile and streams covered with a screen secured to prevent fish escape. Fish were fed twice daily with 1 gram of frozen bloodworms (San Francisco Bay Brand, California, USA). Temperature fluctuated diurnally from 23-28°C (morning to evening), where

25°C is considered ideal for reproducing FHMs, with a natural light regime of around 18hr light and 6 hrs dark. Spawning tiles were checked daily for eggs, collected by rolling the eggs into a petri dish and photographed via a camera mounted to a microscope (Canon Powershot A620, Leica SD 6, magnification 1.0-1.25x). The photograph was used to count the eggs and measure egg size in image sizing software; images were calibrated in the software program using a micrometer that was placed under the petri dish at time photograph was taken (Image Pro Plus 6.1, Media Cybernectics Inc, Maryland, USA). Pre-exposure consisted of 64 trios (8 mesocosm tables with 8 trios per table) and was carried out without exposure to effluent to obtain baseline breeding performance for each trio. After nine days, breeding trios for the exposure period were selected based on the criteria of 100% survival of adults and at least a single breeding event (OECD 2003). In addition, eggs/female/ day (e/f/d) was added as a calculation obtained from the total number of eggs produced divided by the number of females (2) and divided by the total number of pre-exposure days (9). Trios with e/f/d closest to ten were chosen for the remaining streams.

Statistical analyses among the mesocosm tables were conducted at the end of pre-exposure to determine if there were any significant differences in reproductive endpoints before exposure was started. Levene's test was used to assess homogeneity of variance and the Kolmogorov-Smirnoff test (K-S test) to test for normality. One-way Analysis of Variance (ANOVA) was carried out for total eggs and eggs per female per day (e/f/d). No significant differences were found with all p-values > 0.05.

2.2.2 Exposure Design

The exposure portion of the experiment consisted of 4 treatments (8 FHM trios per treatment) in a two-factor design with water and sediment as factors. The treatments were; reference water from Kershner Lake and reference sediment from David Lake (RWRS), reference water and contaminated sediment from Unknown Lake (RWCS), 25% effluent and reference sediment (EWRS) and 25% effluent and contaminated sediment (EWCS). The reference water was taken from Kershner Lake rather than David Lake due to the inability caused by accessibility to collect water in the quantity needed from David Lake. It was deemed to be a suitable substitute as Kershner Lake feeds into David Lake and water quality analysis found them to be similar. Reference sediments were still taken from David Lake, even though

Kershner Lake water was used to maintain consistency with previous sediment work at the site. The use of 25% effluent represented the effluent dilution concentration and the level of Se (approximately 5 µg/L) that has been measured in Unknown Lake (Golder Assoc. Ltd 2005). Contaminated sediments were taken from Unknown Lake. In addition, the sediment used was of low organic content, comprised mainly of fine and coarse sand.

Approximately 11,000L (3,000 gallons) of water were collected from Kershner Lake every 2-3 days, using a truck and trailer system and stored in 2 -1600 gallon tanks on-site co-located with the mesocosm tables. Water from the reference tanks was connected to the mesocosm tables via a distribution tank powered by a March pump (March "Series 3" Seal-less Magnetic Drive Centrifugal Pump Model LC-3CP-MD, John Brooks Co. Ltd. Winnipeg, MB). Treated effluent was taken directly from the mill's treated discharge pipe every three days and diluted using the reference water. There was a turnover of treatment water twice per day in the exposure tables and in the larvae and egg incubation chambers. The water temperature was logged using temperature loggers (Optic Stowaways; Onset Computer, Bourne, MA, USA), and fluctuated diurnally. The mesocosm systems were housed in an opaque tent so both light and temperature followed daily external conditions. In addition, conductivity and dissolved oxygen were recorded daily (YSI meter, Yellow Springs Instruments, Yellow Springs, OH, USA). Ammonia and pH were also recorded daily (Hanna Low Range Ammonia Meter HI93700 and Hanna pH meter, Hanna Instruments Inc., Woonsocket, Rhode Island, USA).

2.2.3 Fathead Minnow Eggs and Larvae

Eggs and larvae produced during the exposure period were held in corresponding treatment tanks with static renewals twice daily. Daily observations of the larvae were recorded to evaluate time-to-hatch, hatching success, 5-day larval survival, and larval deformities. For 5-day survival, each larvae was assessed for deformities and counted as alive or dead. After which the larvae were blotted dry on filter paper and frozen for Se speciation and total metal analyses. Larvae were examined under a dissecting microscope (Leica SD 6, magnification 1.25-2.0x) for gross external deformities and recorded for all broods of larvae. Deformities were recorded as absent or present for each larva. Deformities assessed were scoliosis, lordosis, kyphosis, yolk sac deformities, yolk sac edema, pericardial edema, craniofacial, finfold, hemorrhaging of the body, ocular region, or yolk sac or pericardial region (Rickwood et al. 2006a; 2006b; 2008). For every

brood of eggs laid a subset of 10 eggs were collected as long as there were 20 eggs or more; these were also dried on filter paper and frozen for Se speciation and total metal analyses.

At the end of the exposure, fish were anaesthetized; fork length (mm), total body weight (g), carcass weight (g) and secondary sex characteristics were recorded. Fish were then euthanized by spinal severance, and tails removed. Gonads and livers were removed and weighed; gonads were cut in half and weighed again to ensure equal separation so that total metal and Se speciation analyses could be carried out. Fish bodies were also cut in half for total metal and Se speciation analyses.

All total metal concentrations were determined using inductively-coupled mass spectroscopy (ICP-MS) at SRC Analytical Laboratories in Saskatoon, SK, Canada. Se speciation for all samples except water was conducted at the Hard X-ray Micro-Analysis (HXMA) beamline at the Canadian Light Source (CLS) at the University of Saskatchewan in Saskatoon, SK using X-ray absorption near-edge spectroscopy (XANES) analyses. Tissue samples for speciation were frozen at -80ºC and handled under liquid nitrogen at all times. Samples were ground using an agate pestle and mortar, packed into a frozen cuvette that were sealed with liquid glycerol and frozen using liquid nitrogen. When analyzed at the beamline all spectra were calibrated to 12658.0 eV, the inflection point of the Se K-edge. Speciation methods are described in more detail by Wiramanaden et al. (2010b). Water Se concentrations were below the detection limit at CLS therefore speciation was conducted at Trent University, Petersbourgh, ON by Dr. Dirk Wallschlager. Speciation of water samples was carried out using ion-chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS) methods are given in further detail by Wallschlager and Roehl (2001).

2.2.4 Water, Sediment and Algae Sample Analyses

Water samples were collected from the mesocosm reservoirs three times during the exposure (exposure days 1, 7 and 21) and analyzed for general water quality. Samples were collected, preserved, shipped chilled and analyzed following standardized methods by ALS Laboratory Group, Saskatoon, SK, Canada. General chemistry measurements included: alkalinity (as $CaCO_3$), ammonia, bicarbonate, calcium, carbonate, chloride, conductivity, dissolved organic carbon, hardness, hydroxide, ion balance, magnesium, nitrate, nitrite, pH,

potassium, sodium, sulphate, TDS, total dissolved organic carbon, total nitrogen, and total phosphorus.

Water samples were also taken at the same time as general quality for total metals and Se speciation and frozen on site for later analysis (SRC Analytical, Saskatoon, SK, CANADA and Dr. Dirk Wallschlager, Trent University, Petersbourgh, ON, respectively). Sediment samples were taken from each lake before they were placed into the mesocosms, and collected again from each mesocosm upon completion of the exposure period. Algae/bioiflm samples were also collected from each stream, by collecting scrapings from the sides of the streams at the end of the exposure period. Algae and sediment were analyzed for total metals and Se speciation at the CLS. Sediments were also analyzed for general chemistry and composition for particle size and total organic carbon.

2.2.5 Statistical Analysis

Data were analyzed using SPSS 17.0 (SPSS, Chicago, Il, USA). Homogeneity of variance was tested using Levene's Test and normality using the K-S test. Two-way ANOVA analyses were conducted with water and sediment as the two factors. If normality and variance assumptions were met a two-way ANOVA was performed on hatching success, percent fertilization, larval deformities, larval survival, metal burdens in fish tissues, water, sediment, and algae in addition to water and effluent quality. If parametric assumptions were not met, percentage data were arcsine transformed and non-percentage data were $\log_{10}$ transformed. The two-way ANOVA following data transformation was then performed again on the transformed data, if assumptions held. If the assumptions were still not met then a non-parametric two-way ANOVA test (the Scheirer-Ray-Hare test) was performed. The K-S test was carried out to assess cumulative spawning events and cumulative egg production in each treatment over 21 days compared to the control group.

2.3 Results

2.3.1 Water Quality

In the 25% effluent treatments, water quality showed statistically higher ammonia, conductivity, hardness, chloride, calcium, potassium, magnesium, sodium, and sulphate (two-

way ANOVA, p >0.05). Total nitrogen was also elevated significantly through exposure to effluent and contaminated sediments (two-way ANOVA, p = 0.001 and 0.028 respectively) (Table 2.1). The following metals and metalloids were also elevated in the water with effluent exposure: aluminum, antimony, arsenic, barium, boron, chromium, cobalt, lithium, molybdenum, manganese, nickel, selenium, strontium, thallium, tin, titanium, uranium, and vanadium (two-way ANOVA, p > 0.05). Molybdenum (Mo) was elevated in the water exposed to effluent and this change was dependent upon the presence of contaminated sediment (significant interaction, two-way ANOVA, p > 0.05) (Table 2.2).

2.3.2 Algae Analysis

Increased levels of aluminum, arsenic, beryllium, chromium, cobalt, lithium, rubidium, selenium, uranium and vanadium were found in algae tissues after effluent exposure (two-way ANOVA, p >0.05) (Table 2.3). Several metals showed interesting statistical interactions; that is, the metal increased in algae due to effluent exposure, sediment exposure or a dependent combination of both pathways. Our focus was to identify interactions that are relevant to exposure and thus it is these interactions that are reported. Mo increased in algae after water exposure and was dependent upon the presence of contaminated sediment (significant interaction, two-way ANOVA, p = 0.001 and 0.005) (Table 2.3).

2.3.3 Sediment Analysis

There were no significant differences detected for any of the metals in the reference or contaminated sediments (data shown in Appendix A). Only significant differences were noted in sediment composition between the reference and contaminated sediment. Fine sand (%) was significantly greater in the contaminated sediments (Scheirer-Ray-Hare, p= 0.001). Whereas coarse sand (%) was significantly greater in the reference sediments (Scheirer-Ray-Hare, p = 0.001).

2.3.4 Adult Fish Morphometric Endpoints

Survival, fork length or body mass of adults did not differ between treatments nor did female gonad weight, gonadosomatic index (GSI), liver weight, or liver somatic index (LSI) or male condition factor (CF), gonad weight or GSI. CF in females were 1.25 ± 0.04 (RWRS), 1.34

Table 2.1 Chemistry analysis of reference water (RW) and 25% effluent (EW) in the presence of either reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. Samples taken from one stream per treatment per week for three weeks. Values are mean (n =3) ± standard error of the mean[1].

Variable[2]	**RWRS**	**RWCS**	**EWRS**	**EWCS**
Ammonia	0.03 ± 0.0	0.03 ± 0.0	3.44 ± 0.14 ^{W}	3.21 ± 0.12 ^{W}
Conductivity (µS/cm)	13.3 ± 3.3	10.0 ± 0.0	1046.7 ± 34.8 ^{W}	1033.3 ± 49.1 ^{W}
Hardness	0.7 ± 0.2	0.7 ± 0.2	463.0 ± 23.9 ^{W}	455.7 ± 28.4 ^{W}
pH (pH units)	6.6 ± 0.2	6.7 ± 0.2	6.5 ± 0.1	6.5 ± 0.1
Total Nitrogen	0.5 ± 0.1	0.4 ± 0.1 ^{S}	4.4 ± 0.3 ^{W}	3.9 ± 0.1 ^{W,S}
Sulphate	2.0 ± 0.0	2.0 ± 0.0	491.0 ± 26.5 ^{W}	480.3 ± 31.8 ^{W}
Total Alkalinity	6.3 ± 0.3	6.0 ± 0.0	7.7 ± 0.3 ^{W}	7.3 ± 0.3 ^{W}
Calcium	0.5 ± 0.0	0.5 ± 0.0	174.7 ± 8.6 ^{W}	172.0 ±10.2 ^{W}
Chloride	0.5 ± 0.0	0.5 ± 0.0	15.0 ± 8.6 ^{W}	15.3 ± 10.2 ^{W}
Potassium	0.4 ± 0.1	0.3 ± 0.0	14.6 ± 0.3 ^{W}	14.3 ± 0.3 ^{W}
Magnesium	0.2 ± 0.0	0.2 ± 0.0	6.6 ± 0.8 ^{W}	6.4 ± 0.8 ^{W}
Sodium	0.7 ± 0.2	0.8 ± 0.2	24.7 ± 0.9 ^{W}	23.7 ± 0.9 ^{W}

W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p \leq 0.05$. Analyzed using a two-way ANOVA.

1-Only variables showing significant difference are presented

2- All units in mg/L unless otherwise indicated

Table 2.2: Total metal water analysis reference water (RW) and 25% effluent (EW) in the presence of either reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. Samples taken from one stream per treatment per week for three weeks. Values are mean (n =3) ± standard error of the mean[1].

Variable[2]	**RWRS**	**RWCS**	**EWRS**	**EWCS**
Al	11.8 ± 2.5	16.0 ± 4.5	87.1 ± 7.0 ^{W}	83.7 ± 18.8 ^{W}
Sb	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0 ^{W}	0.3 ± 0.0 ^{W}
As	0.6 ± 0.2	0.6 ± 0.1	7.1 ± 1.6 ^{W}	5.5 ± 1.6 ^{W}
Ba	3.2 ± 0.4	2.4 ± 0.0^{S}	23.0 ± 0.0 ^{W}	22.3 ± 2.5 $^{W, S}$
Bo	10.0 ± 0.0	10.0 ± 0.0	300.0 ± 30.0 ^{W}	280.0 ± 30.0 ^{W}
Cr	0.3 ± 0.0	0.3 ± 0.0	3.0 ± 2.0 ^{W}	4.9 ± 2.0 ^{W}
Co	0.1 ± 0.0	0.1 ± 0.0	1.7 ± 0.1 ^{W}	1.7 ± 0.1 ^{W}
Cu	12.3 ± 0.3	2.3 ± 0.2 ^{S,I}	6.1 ± 1.3	5.1 ± 1.5 ^{S,I}
Li	1.0 ± 2.0	0.9 ± 0.2	183.3 ± 23.3^{W}	206.7 ± 29.1 ^{W}
Mn	1.8 ± 0.6	1.3 ± 0.2	3.9 ± 0.6 ^{W}	4.4 ± 0.4 ^{W}
Mo	0.2 ± 0.1	1.0 ± 0.2	215.3 ± 47.7 ^{W}	187.3 ± 39.9^{W,I}
Ni	2.0 ± 0.4	0.6 ± 0.0	31.7 ± 0.3 ^{W}	35.0 ± 4.0 ^{W}
Rb	0.8 ± 0.0	0.7 ± 0.0	37.9 ± 1.5 ^{w}	38.9 ± 1.3^{w}
Se	0.1 ± 0.0	0.1 ± 0.0	10.4 ± 1.6 ^{W}	9.0 ± 4.0 ^{W}
Sr	11.0 ± 0.6	10.1 ± 0.5	260.0 ± 10.0^{W}	286.7 ± 23.3 ^{W}
Tl	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.1 ^{W}	0.4 ± 0.1 ^{W}
Sn	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.3 ^{W}	0.1 ± 0.0 ^{W}
Ti	0.2 ± 0.1	0.1 ± 0.0	1.4 ± 0.4 ^{W}	1.2 ± 0.6 ^{W}
U	0.2 ± 0.1	0.1 ± 0.0	1.4 ± 0.4 ^{W}	1.2 ± 0.6 ^{W}
V	0.1 ± 0.0	0.1 ± 0.0	1.5 ± 0.4 ^{W}	2.9 ± 1.0 ^{W}
Zn	20.7 ± 8.3	4.6 ± 0.9 ^{S}	16.7 ± 1.7	10.1 ± 0.5 ^{S}

W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p \leq 0.05$. Analyzed using a two-way ANOVA.

1-Only variables showing significant difference are presented

2- All units in µg/L unless otherwise indicated.

Table 2.3: Metal algae analyses of reference water (RW) and 25% effluent (EW) in the presence of either reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. Three samples taken from each treatment following exposure. Values are mean (n =3) ± standard error of the

Variable[2]	**RWRS**	**RWCS**	**EWRS**	**EWCS**
Al	75.0 ± 18.9	55.7 ± 15.2	179.0 ±50.3 [W]	145.3 ± 39.4 [W]
As	0.24 ± 0.06	0.15 ± 0.02	1.00 ± 0.23 [W]	0.67 ± 0.13 [W]
Be	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00 [W]	0.01 ± 0.00 [W]
Cd	0.03 ± 0.01	0.01 ± 0.00 [S]	0.02 ± 0.00	0.01 ± 0.00 [S]
Cr	0.3 ± 0.0	0.3 ± 0.0	2.3 ± 0.5 [W]	1.8 ± 0.4 [W]
Co	0.04 ± 0.01	0.02 ± 0.00	0.76 ± 0.27 [W]	0.71 ± 0.10 [W]
Cu	24.33± 6.23	1.50 ± 0.15 [S, I]	11.90 ± 2.72	2.73 ± 0.74 [S. I]
Fe	175.3 ± 51.7	80.3 ± 12.5 [S]	427.0 ± 102.0 [W]	178.0 ± 37.5 [W,S]
Pb	0.63 ± 0.16	0.15 ± 0.03 [S]	0.58 ± 0.11	0.29 ± 0.06 [S]
Li	0.08 ± 0.02	0.06 ± 0.01	0.51 ± 0.12 [W]	0.41 ± 0.07 [W]
Mo	0.1 ± 0.0	0.1 ± 0.0	1.3 ± 0.1 [W, I]	0.6 ± 0.2 [W, I]
Ni	1.69 ± 0.41	0.36 ± 0.05 [S,I]	3.87 ± 1.20 [W,I]	2.30 ± 0.26 [W,S,I]
Rb	0.247 ± 0.066	0.260 ± 0.026	0.527 ± 0.101 [W]	0.383 ± 0.049 [W]
Se	0.03 ± 0.00	0.03 ± 0.00	0.55 ± 0.12 [W]	0.48 ± 0.07 [W]
U	0.09 ± 0.02	0.07 ± 0.01	2.87 ± 0.58 [W]	2.43 ± 0.33 [W]
V	0.2 ± 0.1	0.1 ± 0.0	1.9 ± 0.5 [W]	1.1 ± 0.2 [W]

W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p \leq 0.05$. Analyzed using a two-way ANOVA.

1-Only variables showing significant difference are presented

2- All units in µg/g wet weight unless otherwise indicated

± 0.04 (RWCS), 1.44 ± 0.06 (EWRS), 1.44 ± 0.06 (EWCS) and showed a significant interaction after water and sediment exposure (Scheirer-Ray-Hare, p =0.008). LSI in males was 0.019 ± 0.003 (RWRS), 0.021 ± 0.003 (RWCS), 0.015 ± 0.001 (EWRS), 0.013 ± 0.003 (EWCS) after effluent exposure (2-way ANOVA, p = 0.036). The pattern also occurred for liver weight in male FHMs (2-way ANOVA, p = 0.031).

2.3.5 Reproductive Output

There were significant increases in cumulative spawning events for the effluent treatments (Kolmogrov-Smirnoff, $p < 0.05$) (Figure 2.2). There were also significant increases in total cumulative egg production and cumulative eggs/female day in the effluent treatments (Kolmogrov-Smirnoff, $p < 0.05$) (Figure 2.3). There was no significant change in percent egg fertilization between treatments. Hatching success significantly decreased after effluent and sediment exposure although there was no interaction shown between statistical factors (2-way ANOVA, p= 0.001 and 0.044 respectively) (Figure 2.4a). Egg size significantly decreased with effluent exposure (Scheirer-Ray-Hare, p=0.001). Eggs that hatched were assessed for deformities and 5-day survival. There was a significant 20% decrease in larval survival in the effluent treatments (Scheirer-Ray-Hare, p=0.001) (Figure 2.4b). Edema (yolk sac and pericardial) was kept separate during analysis of deformities as the persistence and potential effects of these abnormalities on long term survival are not known and may be reversible. The percentage of deformities increased by approximately six-fold in the effluent treatments with the changes dependent upon sediment exposure as indicated by a significant interaction (Scheirer-Ray-Hare, p=0.001 and 0.023 respectively). There were 6.81% deformed embryos in EWRS and 10.24% deformed in EWCS, with skeletal deformities predominating in all effluent and reference treatments (Figure 2.4c). Other types of deformities included larvae with no body and/or tail development, and presence of unexplained large white areas on the body especially around the spinal and head regions of live larvae. There was a significant effluent effect on the days to hatch for the larvae with eggs in the EWRS treatment hatching faster and after only 1-2 days post spawn. Analysis by two-way ANOVA found a significant sediment effect and interaction for days to hatch (Scheirer-Ray-Hare, p=0.016 and 0.001 respectively).

The effects on larvae were examined for each treatment as the proportion of normal larvae hatched per trio. The mean proportions were 0.94 ± 0.05 (RWRS), 0.97± 0.04 (RWCS), 0.69 ±

0.32 (EWRS) and 0.73 ± 0.28 (EWCS) (Figure 4d). A significant decrease was noted in the proportion of normal larvae per trio following effluent exposure (two-way ANOVA, $p < 0.05$) (Figure 2.4d). There was however no significant difference (two-way ANOVA, $p > 0.05$) noted when the mean number of normal larvae per trio was compared. Results were as follows: RWRS 158.9, RWCS 118.2, EWRS 200.4 and EWCS 144.9 mean normal larvae per trio (Figure 2.4e).

2.3.6 Tissue Burdens

Tissue values are reported as a converted dry weigh based on an assumed 80% moisture content. Female muscle tissue showed significant increases in arsenic, cobalt, iron, lithium, molybdenum, rubidium, selenium, uranium and vanadium after effluent exposure (two-way ANOVA, $p < 0.05$). Statistical increases in mercury were detected for effluent and sediment exposure but no interaction was detected (Scheirer-Ray-Hare, $p= 0.001$ (water) and $p= 0.031$ (sediment)). Barium (Ba) in female muscle was elevated after contaminated sediment exposure (two-way ANOVA, $p = 0.001$). Cadmium (Cd) was significantly elevated in female muscle tissue exposed through contaminated sediment with a dependence on water exposure (two-way ANOVA, $p = 0.011$ and 0.047).

Female ovaries showed significant increases in arsenic, cobalt, rubidium, and selenium when exposed to the water (two-way ANOVA, $p< 0.05$). Uranium (U) levels were elevated after water exposure with an interaction reported suggesting dependence on sediment exposure (two-way ANOVA, $p = 0.001$ and 0.013). Egg samples were pooled to meet tissue needs for metal analyses. Increases were noted following water exposure for mercury, nickel, rubidium, selenium, uranium and zinc (two-way ANOVA, $p < 0.05$). Five day old larvae samples were pooled to meet tissue volume needs. There was a significant increase for Se following water exposure (two-way ANOVA, $p < 0.05$). Cu and rubidium (Rb) were significantly increased after exposure to effluent and contaminated sediment (two-way ANOVA, $p < 0.05$). Additionally, there was a significant increase in strontium (Sr) and zinc following exposure to contaminated sediment with an interaction reported for Sr (two-way ANOVA, $p < 0.05$).

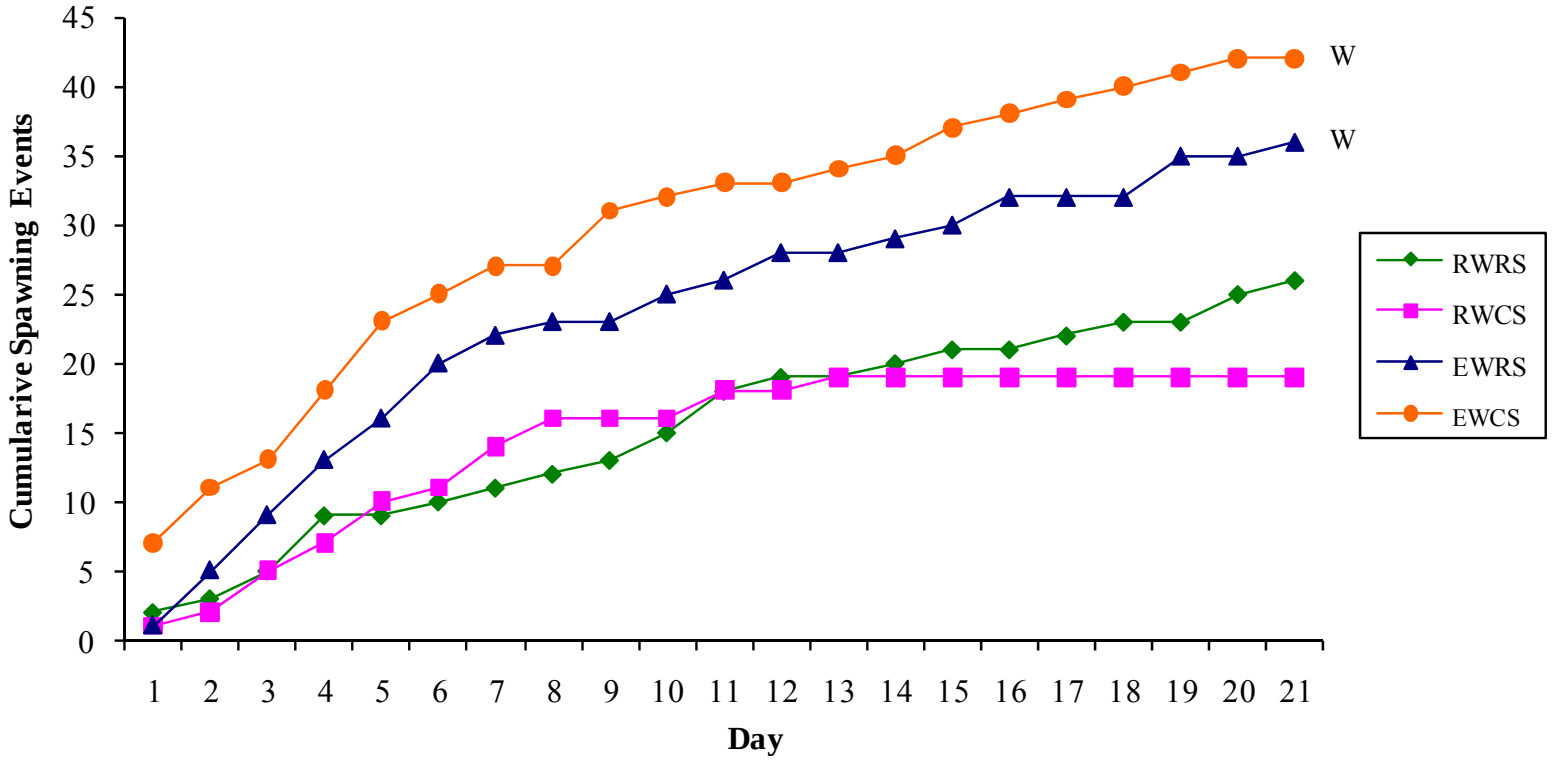

Figure 2.2: Cumulative spawning events of fathead minnow trios exposed to reference water (RW) or 25% effluent (EW) in the presence or absence of reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p < 0.05$.

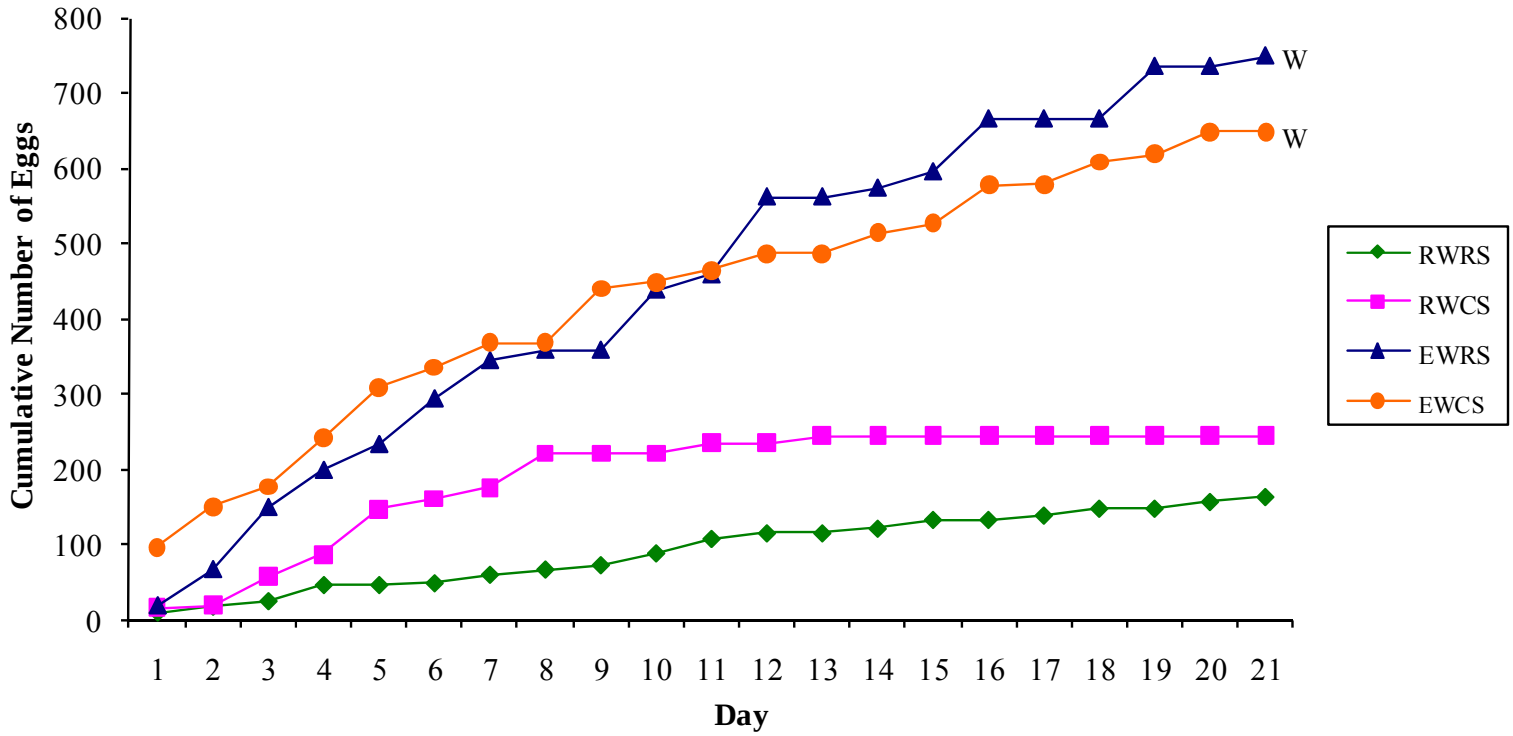

Figure 2.3: Cumulative egg production of fathead minnow trios exposed to reference water (RW) or 25% effluent (EW) in the presence or absence of reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p < 0.05$.

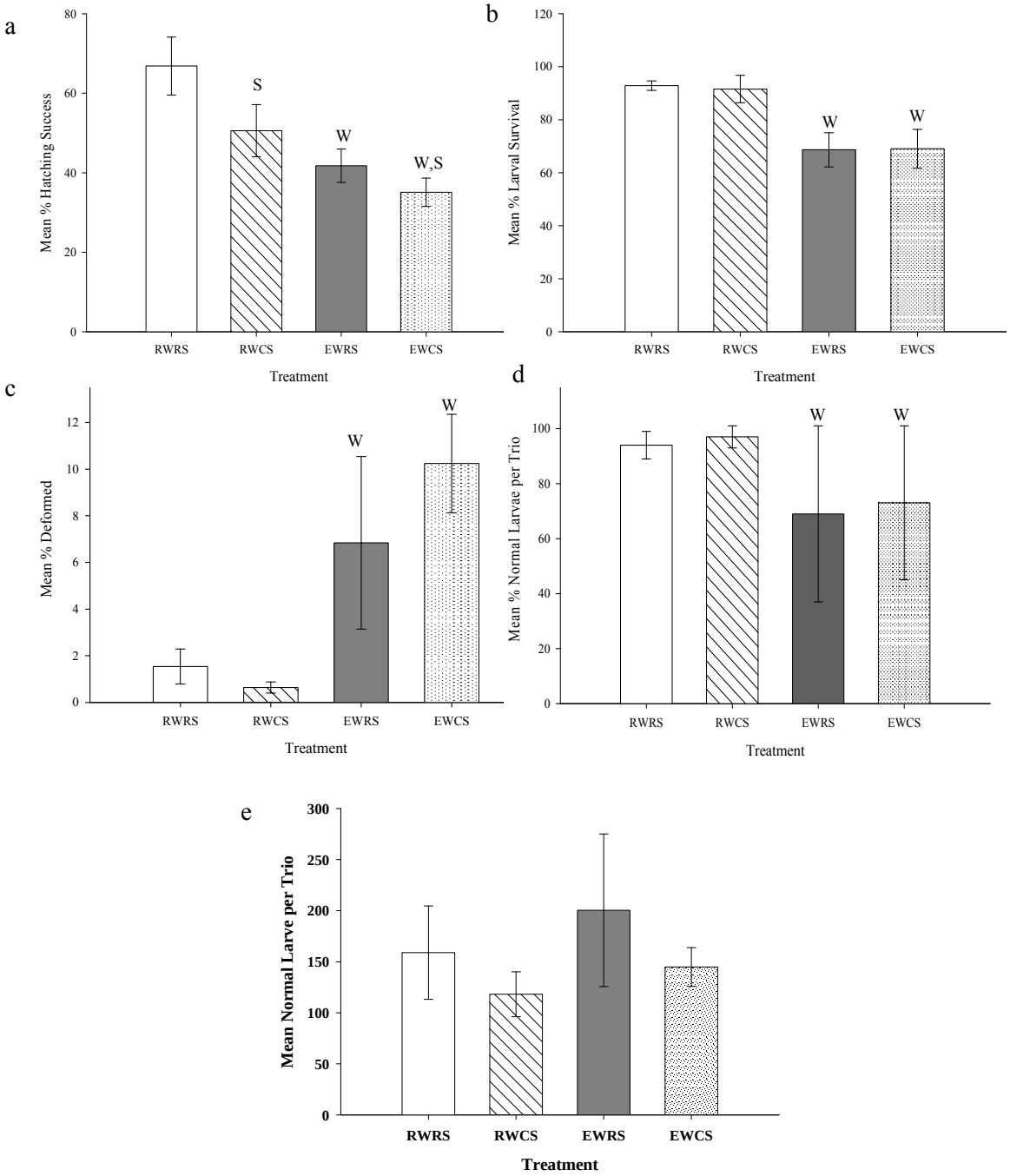

Figure 2.4: FHM egg and larval endpoints (a) hatching success of eggs (b) larval survival to 5-days post hatch (c) larval deformities assessed after 5-day post hatch (d) mean proportion(%) of normal larvae per trio and (e) mean number of normal larvae per trio exposed to reference water (RW) or 25% effluent (EW) in the presence or absence of reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. Error bars represent standard error. W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p < 0.05$.

Se was elevated in muscle, ovaries, eggs and larvae of fish (Figure 2.5) therefore whole-body Se levels were calculated for female FHMs using the standard equations in the United States Environmental Protections Agency (USEPA) Se guidance documents (USEPA 2004). Whole body was calculated using concentrations of Se in the ovaries on a dry weight basis. Whole body concentrations were calculated as 4.65 µg/g (RWRS), 4.11 µg/g (RWCS), 7.90 µg/g (EWRS), and 9.60 µg/g (EWCS). From this calculation, the concentration of Se deposited into the eggs daily was estimated using the mean Se egg concentration and the mean total eggs/female/day value. It was found that there was 0.046 µg (RWRS), 0.032 µg (RWCS), 0.073 µg (EWRS) and 0.078 µg (EWCS) of Se deposited on average into the eggs daily. The proportion of the adult Se body burden deposited into the eggs was similar across treatments 0.010 (RWRS), 0.008 (RWCS), 0.009 (EWRS) and 0.008 (EWCS).

2.3.7 Speciation

Speciation analysis for Se was conducted on water, algae, female muscle, female ovaries, eggs and larvae. Female muscle showed a decrease in the proportion of selenocystine-like compounds (R-Se-Se-R) and an increase in selenomethionine-like compounds (R-Se-R) with effluent exposure. RWRS and RWCS had similar Se profiles with both exhibiting over 80% R-Se-Se-R. EWRS was 71% R-Se-Se-R and 19% R-Se-R; EWCS was 46% R-Se-Se-R and 44% R-Se-R. All samples except RWCS had small proportions of selenite between 5-8%. Female ovaries showed similar results with decreasing proportions of R-Se-Se-R with increases in the proportion of R-Se-R. For the egg speciation data, RWRS, RWCS and EWRS all appear to be similar in their proportion of R-Se-Se-R (53-61%) and R-Se-R (37-42%). The reverse is noted in EWCS with 53% R-Se-R and 42% R-Se-Se-R. Larvae results indicate that RWRS and EWCS are similar with greater than 80% R-Se-Se-R and 5-11% selenite. RWCS and EWRS are comparable with 53% and 68% R-Se-Se-R and 35% and 28% R-Se-R respectively (Figure 2.6a-d). Algae had insufficient sample volume to obtain results for RW treatments. EWRS and EWCS algae samples had high proportions of R-Se-R (65% and 50% respectively). Both treatments also contained additional forms not seen in the other tissues and differed from each other. EWRS indicated elemental Se 11%, selenite 10%, and MSe 9% and EWCS showed metal selenide (MSe) 27%, seleno-diglutathione (GSe, R-S-Se-S-R) 11%, and dimethyl seleno oxide (DMSeO) 10%.

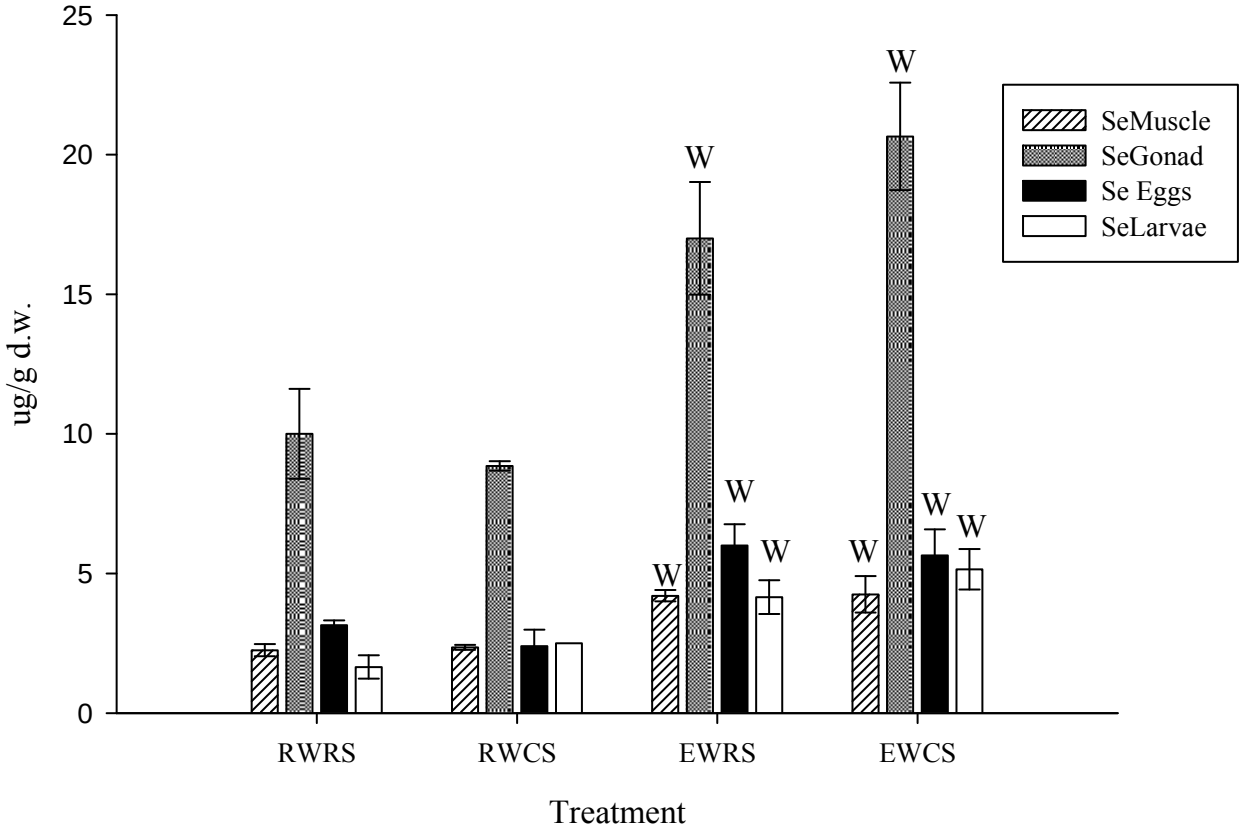

Figure 2.5: Concentration of Se (µg/g dry weight) in FHM female muscle and ovaries, and FHM eggs and larvae exposed to reference water (RW) or 25% effluent (EW) in the presence or absence of reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. Error bars represent standard error. W indicates a water effect, S indicates a sediment effect and I indicates an interaction, where $p < 0.05$.

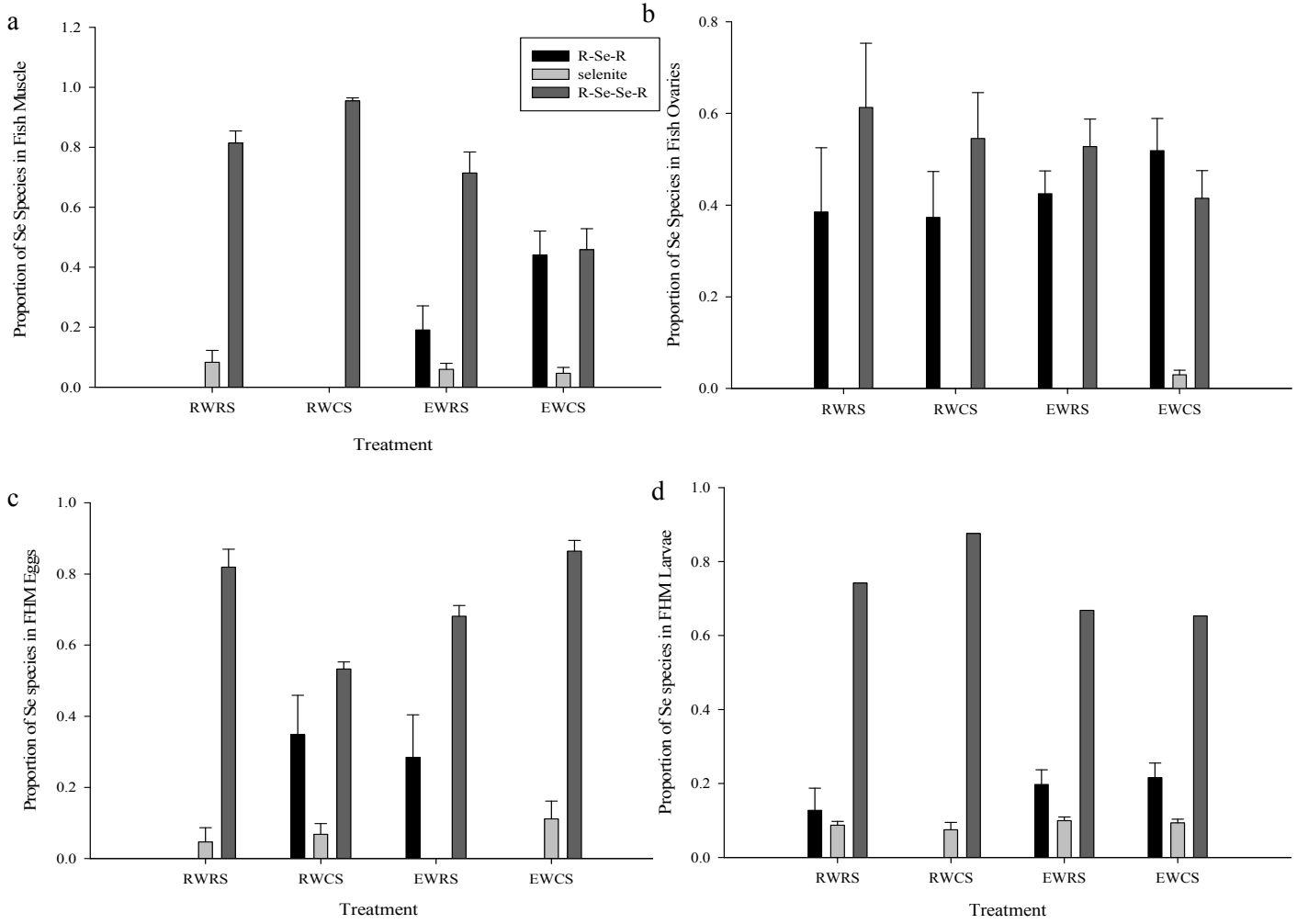

Figure 2.6: Speciation of FHM tissues with the relative proportion of R-Se-R, selenite and R-Se-Se-R of the total Se concentration (a) Female FHM muscle (b) female FHM ovaries, (c) FHM eggs (d) FHM larvae exposed to reference water (RW) or 25% effluent (EW) in the presence or absence of reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams.

Analyses of the water in the reference treatments showed very low concentrations of Se and speciation results were below the detection limit for selenate and selenite. For the effluent treatments, three EWCS and one EWRS samples showed a predominance of selenate. In contrast the two other EWRS samples showed selenite as the main form of Se in the water. When the effluent is discharged the predominant form found is selenate, therefore we are seeing transformation in the exposure water.

2.4 Discussion

The objectives of this study were to assess the effects of treated uranium milling effluent on FHM health and reproduction (e.g., spawning events, total eggs produced) using on-site mesocosm systems to provide increased environmental relevance compared to laboratory exposure studies. By using the FHM partial lifecycle reproductive bioassay as modified by Rickwood et al. (2006a,b; 2008) the effects of milling effluent, in the presence and absence of sediment contamination, on reproduction and survival of the adults and larvae were evaluated. The project examined the effects of whole effluent, diluted to an environmentally relevant 25% concentration as well as placing an emphasis on the movement and accumulation of Se in water, sediment, algae and fish through speciation analyses. Understanding the routes of exposure (water versus sediment versus both) is critical to inform regulatory processes and to guide further research on fate, effects and potential mitigation.

Research examining metal mine effluent effects on fish exposed to metals, have reported increased liver size, decreased egg size, reduced growth and decreased condition factor (Weber et al. 2008; Bervoets et al. 2005). Changes in condition factor or liver indices may indicate a depletion of energy reserves or disruption of metabolic processes and has been reported with exposure to metals (Munkittrick and Dixon 1988; Levesque et al. 2002). In our study, effluent exposure resulted in lower LSI and liver weight for male FHM. Decreases in liver size for male fish have been noted in a previous monitoring work as part of the Environmental Effects Monitoring (EEM) program (Golder Assoc. Ltd 2005). Other researchers that sampled feral northern pike found no significant difference in LSI for fish taken from David Lake (reference) and Unknown Lake (exposure) (Kelly and Janz 2008). The decreases in LSI for this study could be due to several factors. Research examining the effects of uranium exposure to larval white suckers found spotting on male and female livers (Liber et al. 2004). Liver spotting was not

noted in this study but the decrease in male LSI could be from metal exposure affecting the liver tissue. Lemly (2002b) also reported that exposure to Se can lead to degradation of tissue structure and could alter liver function in exposed fish which may account for our decrease in LSI and liver weight. A decrease in food abundance, the ability to metabolize food resources, or increased energy use for detoxification may also be factors. The former is not a factor in our study as fish were fed a controlled diet (approximately 1 μg/g d.w Se) of equal amounts amongst treatments. We can only speculate there may have been changes in male energetics as no biochemistry analyses or liver histology was conducted for this study. Also, female FHM condition factor increased with effluent exposure with water and sediment factors showing significant interactive effects. Whereas, Kelly and Janz (2008) did not report any affects on CF for feral fish in the exposure lakes.

The most notable changes in our study were those of effluent exposure on reproductive output and larvae. Fish trios in the effluent exposures experienced increased egg production in terms of both mean eggs produced and total eggs produced. One hypothesis is that the increase in egg production in the effluent treatments may be attributed to the reference water being naturally low in hardness, pH and nutrient levels and the addition of the effluent increased mineral and nutrient levels and stimulated egg production. Hardness and pH are both below those reported for accepted ranges for culturing FHM. Our reference values for hardness were 0.7 ± 0.2 mg/l $CaCO_3$ and pH was 6.6 ± 0.2 and reported ranges for FHM are 45 mg/l $CaCO_3$ for hardness and pH 7.5-8.1 (Jensen et al. 2001). Low dose stimulation of egg production in FHM has also been reported by Rickwood et al (2006a, 2008); for metal mine and pulp and paper effluents. It is also possible that a hormesis effect may be occurring after exposure to the effluent. This biphasic-dose response relationship is commonly seen with essential nutrients and includes a low-dose stimulation and high-dose inhibition (Calabrese 2008). Se is an essential nutrient so it is possible that the 25% effluent provided a more optimal Se level or nutrient/ion levels than what is available in the reference water (Dobbs et al. 1996). One key note is that this study used two concentrations (0% and 25%) and thus an appropriate dose-response relationship cannot be established nor was it the intent of the experiment. However, other published (Rickwood et al. 2006b) and unpublished research from our lab illustrate low dose stimulation of egg production is commonly observed. Thus, this hypothesis of a hormetic response should be considered when designing future studies (Calabrese 2008).

Research using a multi-trophic application of the mesocosm systems suggested that increased egg production and spawning events may lead to eggs of inferior quality (Rickwood et al. 2008). However, that experiment did not see a significant difference in hatching success or egg size. The eggs from our study in the effluent treatments were significantly smaller in size and experienced reduced hatching success which may indicate that though more eggs were produced, they were of poorer quality. Se is known to be deposited into eggs by adult female FHM (maternal transfer); they may have been producing more eggs as a means to reduce their elevated selenium body burden and possibly other metals (SETAC Pellston 2009). The amount of Se deposited daily into the eggs from the female body burden was estimated. From this it can be seen that more Se was deposited into the eggs of effluent exposed fish. The values were on average almost double of those exposed to reference water. However, the proportion of Se deposited based on the female's body burden was similar across treatments. More Se was deposited into the effluent treatments eggs, but since the brood sizes were larger in EW the proportion of Se was comparable across treatments.

The early hatch of eggs in this study coincides with previous work at Key Lake that has also noted early hatch in FHM eggs that were held in lakes near the mill, these were Little McDonald and McDonald Lake that received Deilman open-pit dewatering effluent (Pyle et al. 2001). Those findings indicated that eggs placed in high Ni and low hardness water experienced early time to hatch. The FHM eggs produced in this mesocosm study were exposed to increased levels of Ni but in a high water hardness environment due to the nature of the milling effluent. Similar work has shown that high Ni levels and high hardness can also lead to early hatch (Pyle et al. 2002a). Ni has also been shown to affect time to hatch in zebra fish eggs, but in those instances Ni levels at and above 40 µg/L exposure delayed hatching (Dave and Xiu 1991). Levels of Ni in the present study were around 35 µg/L and could have had an impact on the observed early hatch, but the levels are generally lower than those at Little McDonald and McDonald Lake, 116.01 ± 9.12 µg/l and 53.56 ± 20.83 µg/l respectively, with some overlap. Another possibility is that FHM eggs have been reported to be sensitive to Cd exposure in the water though there have been contradicting reports on its linkage to early or delayed hatch (Pickering and Gast 1972; Gauthier et al. 2006). However, our levels in the water for Cd were low around 0.02 µg/L and not likely to affect hatching on its own but may play a role as the effluent is a mixture. Another factor affecting hatch could be a fungal or bacterial infection as

natural water and sediment were used, the eggs would certainly have been exposed to infectious agents. Occurrence of fungal-infected eggs were noted and number infected recorded in all treatments although there was no statistical difference and not likely to play a large role in the observed effects. It has been reported that infectious agents can lead to early hatch in whitefish eggs while it has also been reported that infection has no effect on egg hatching for sardines (Wedekind 2002; Miquez et al. 2004).

Increases in the incidences of larval deformities in the effluent exposures also support the maternal transfer nature of Se (Schultz and Hermantuz 1990). Research at Key Lake has found increases in deformities of northern pike larvae at a medium exposure site; Delta Lake which is downstream of Unknown Lake (Muscatello et al. 2006). Based on hydrological data, Delta Lake can be considered to receive an effluent concentration of about 5% of the levels of Se in the 100% effluent (Golder Assoc. Ltd 2005). Teratogenic deformities of the hard and soft tissues of larval fish are good indicators of Se toxicity and are predominately present as skeletal (lordosis, scoliosis), fin-fold, and craniofacial (Lemly 1997a). For our analyses of deformities, edema was not included as it can be reversible in some situations and the skeletal deformities are more commonly associated with Se exposure. However, it should be noted that edema, bulging eyes and cataracts though not deformities, may still be an indication of Se toxicity (Lemly 1997a).

The survival of the larvae to 5-days post hatch was significantly decreased in the effluent treatments by 23% relative to controls. Correlations have been found between larval mortality and Ni and Cd in a study conducted on lakes in Sudbury, Ontario, Canada. Mortality in that was study was between 5-16% with the highest being 43%. However, our levels of Ni and Cd were below those reported for the lakes in the Sudbury area (Gauthier et al. 2006). Similar analysis on larval survival/mortality from a study using FHM larvae caged in the exposure lakes at near-edge sites, which tend to be higher in sand, at Key Lake (Pyle et al. 2001) showed Fox and Unknown Lakes had significantly higher mortality rates (90%) after a 7-day exposure, 70% greater than that observed at the reference locations. They found that Mo correlated strongly with mortality however, laboratory studies with Mo has found it to be relatively nontoxic to fish after exposure to levels well above those observed at Key Lake. Pyle et al. (2001) also found correlations between larval mortality and As, Cd, Cu, Hg, Fe, Se, and V, many of which were found to be elevated in this study. The metals As, Fe, Hg and V were below the values reported by Pyle et al.

(2001) where as Cu and Se were greater in the current study. Values were 6.1±1.3 µg/L and 5.1±1.5 µg/L for Cu and 10.4±1.6 µg/L and 9.0±4.0 µg/L for Se compared to 0.88±0.15 µg/L for Cu and 4.51±0.77 µg/L for Se. It is possible that Se is not the only factor that may be affecting larvae mortality in these systems though it is believed to be the primary contributor. Although we see these impacts on eggs and larvae following exposure it is still noted that the mean number of normal larva per trio was still comparable among all treatments when egg production levels were considered.

Some effluent effects on the adults were observed. However, the primary and more consistent responses involved eggs and larvae. The US EPA Water Quality Criteria for Se indicates, "A reduction of 20 percent in the response observed at control (EC20) was used as the chronic value" (US EPA 2004). It can be hard to define a specific percent change as ranges of 5-30% have also been mention depending on species difference and endpoints (US EPA 2004, Muscatello et al. 2006). The use of a comparison of 20% change from reference or control levels has been considered here as previous work at this site with northern pike work has also used this proposed 20% (Muscatello et al. 2006). For hatching success, the decrease was approximately 23% between the RWRS treatments and both effluent treatments. Larval survival to 5-days was reduced by 31% between RWRS and both effluent exposures; also interpreted as larval mortality increased by 31%. As per Muscatello et al. (2006), we can also choose larval deformities as a response variable and compare to the proposed 20% change threshold. Deformities in the control (RWRS) treatment were 1.64% compared to 6.81% and 10.24% (without edema) in the effluent treatments. These levels fall below the 20% threshold. When edema was included in the calculation of deformities for comparison to Muscatello et al. (2006), slight increases were noted of 8.46% in EWRS and 10.71% in EWCS; changes still below the proposed 20% guideline. Inclusion of edema in the RS treatments did not change the percent deformities among larvae. Other larvae research at Key Lake examined northern pike fry and reported increases of 19% and 27% in the incidence of deformities (edema included) at sites exposed to the effluent compared to reference sites (Muscatello et al. 2006). The differences in the incidence of deformities between the studies could be attributed to different species sensitivity for FHMs and pike (Chapman 2007), and the shorter exposure of the FHMs (21 days) compared to that of northern pike (potentially life-long).

A possible implication of shorter exposure is suggested by total Se concentrations in the eggs and muscles which were higher in the pike study (potential life-long exposure) than in the FHM mesocosm study (21 day exposure). This could indicate that our lack of a natural dietary source, such as exposed benthic invertebrates, as would occur in the lakes, did not allow for as much uptake into the fish as was seen in the feral fish study by Muscatello et al. (2006). It could also suggest that the duration of our exposure may not allow for steady state in the FHM, and that since Se bioaccumulates, the collection of eggs from northern pike could be higher as adults would have been feeding off dietary sources in the increased Se environment (Lemly 1985).

Significantly elevated levels of Se and other metals were present in all samples except sediment most likely due to the low organic content of the sediment. Since there was little organic content there was little metal binding expected and confirmed with no significant difference being detected. Sediment seemed to play a minimal role in observed effects, due to the sediment being primarily sand. Sediments were collected near the shore and represent areas that FHM adults and larvae would normally be found (Pyle et al. 2001). Sediment composition was slightly different between exposed and reference, with a higher percentage of fine sand at the exposed sites.

Dietary Se exposure is a major route of exposure, and this study lacked a dietary exposure route as our intent was to specifically isolate waterborne exposure from sediment. Fish were observed grazing on the algae in all treatments and Se was accumulated in all fish tissues following effluent exposure. As it has been recognized that algae/biofilm plays an integral role in moving and altering the speciation of Se from the water column into the food web, further work incorporating this trophic level into the mesocosm studies for controlled experimentation would be desirable. Algal/biofilm mechanisms of Se movement have not been clearly defined but are also noted in work by Wiramanaden et al. (2010a,b).

The Se speciation results in the tissues showed increased R-Se-R (selenomethionine like compounds) with increasing effluent exposure, whereas the proportion of R-Se-Se-R (selenocystine like compounds) stayed fairly constant with changes in exposure. This would be expected as R-Se-Se-R is more highly regulated in the body then R-Se-R so the level of R-Se-R would be more likely to fluctuate with changes in exposure (Suzuki 2005). For the water analyses since selenite was detected in samples collected from the EW treatments, we do know

that transformation was occurring in the systems, as selenate is the released form of Se in the discharged effluent (Wiramanaden et al. 2010a).

Se has been the primary focus of the work at Key Lake but there were significant increases in other metals in the water, algae, and fish tissue samples consistent with a complex mixture exposure. Since endpoints of interest were reproductive, other potential metals of interest are those that were significantly increased in the female muscle, ovaries, eggs and/or larvae. Thallium, tin, titanium, antimony, and boron were elevated in the water and were not found to be elevated in the tissues analyzed suggesting little effect on observed responses. Similarly aluminum, beryllium, and chromium were only elevated in the water and/or algal fractions and not in the fish tissue.

A trend of detectable amounts of Rb in the water, algae and in fish tissues was noted in this study. Very little is known about the effects of Rb on fish or even at what concentrations any effects may occur. Research examining spermatogenesis in male cultivated catfish (*Pangasiandon hypophthalmus*) reported a correlation between a decrease in GSI and increasing Pb, Rb, As and Mo exposure (Yamaguchi et al. 2007). They also found that Japanese eel (*Anguilla japonica)* testicular cell lines did not respond when exposed to each metal alone but when exposed to all metals there was a decrease in spermatogenesis (Yamaguchi et al. 2007).

The level of mercury (Hg) was elevated in the fish muscle and egg tissue for the effluent treatments. Klaverkamp et al. (2002) reported that muscle tissue of fish was a good indicator of exposure to Hg in the form of methyl mercury due to the lipid soluble nature of this organic form of mercury. We do not know the form of mercury in the present study but a significant increase in Hg in muscle tissue of FHM (RWRS 0.27 µg/g, RWCS 0.22µg/g, EWRS 0.34 vg/g and EWCS 0.39 µg/g w.t) was observed. FHM exposed to dietary methyl mercury at levels above 0.88 µg/g d.w. experienced delayed spawning and decreased spawning success, reduced fecundity and GSI (Hammerschmidt et al. 2002). This is in contrast to the effects we saw in FHM exposed to 25% effluent and levels in the diet were 0.125 µg/g d.w for Hg, therefore we would suggest that Hg is not a major player in this system. However, Hg is known to interact with Se often affecting observed toxic effects (Peterson et al. 2009). Again, causality is difficult to assess when examining metals on their own when they are part of a complex effluent mixture.

Yamaguchi et al (2007) revealed the complexities of interpreting potential causative factors affecting biological responses after exposure to complex mixtures. Due to the complexity of mixtures, the tendency in many studies is to focus hypothesis testing on specific metals of interest despite the receiving environment being exposed to the effluent as a whole. While this may focus the experimental design, the conclusions can be misleading and of lower relevance to the actual "real" environment as no single mine or mill discharges a single metal or element. Based upon the experience in our laboratory with multiple effluent mixtures (Dube and MacLatchey 2002a; Hruska and Dubé 2004; Dubé et al 2006; Rickwood et al. 2006c) we strongly recommend any investigations of whole effluent mixtures include a treatment of exposure to the mixture so any single metal treatments can be compared to the mixture response. A failure to do so lends itself to simplifying a mixture and its potential regulation to a few single metals which may not hold sole responsibility for the observed effects.

Finally, the primary objective of this research was to examine a treated whole effluent mill mixture and determine if exposure to the mixture affected FHM health and reproduction and if the effects manifested through a waterborne pathway of exposure, sediment-borne or were dependent upon both pathways. We paid closer attention to Se in this study as a potential contaminant of concern based upon previous research at this site and the interest in Se globally as an environmental contaminant. This study was a "spring board" to focus subsequent research on specific comparisons between the whole effluent mixture and Se as a potential causative constituent. Our study showed that the water pathway was more significant than the sediment pathway of exposure. The composition of the sediment was sand which represented the largest spatial extent of the study area. Further research to evaluate the organic portion of the sediment is important due to the known interactions between Se binding and organic matter. Although this sediment type is far less abundant at the study site, understanding its direct contribution to fish reproductive effects would benefit a more complete assessment of potential risk and contribution.

2.5 Conclusion

This study demonstrated a variety of reproductive effects on FHM exposed to a complex uranium milling effluent in a mesocosm system. The observed effects were attributable almost entirely to effluent exposure rather than sediment exposure. Further studies to assess effects due

to other sediments, especially those of higher organic carbon content from the location may be of interest. In addition, further work examining the role of algae/biofilms in the movement of Se from the aqueous phase into fish tissues will be integral in developing a clearer understanding of the dynamics of Se in the environment. Understanding the role of Se when discharged via a complex mixture is not straightforward. Caution is necessary to ensure any assessments of cause due to a single metal is done with the full understanding of the complexities of the mixture; the latter being of the greatest relevance for regulatory practice. Our study shows that at this site and at the time of this study, the current treated effluent discharge affected FHM to a much greater extent than effects due to sand sediments exposed to the effluent for a lengthy period of time (historical contamination). In the absence of the mesocosm system the ability to separate out effluent from sediment effects in a replicated longer term reproductive study with fish would have been limited.

Chapter 3:

Correlation analysis and Principal Component Analysis of selenium and rubidium tissue residues

This chapter will be submitted to Integrated Environmental Assessment and Management under joint authorship with Monique Dubé and Paul Jones.

3.1 Introduction/Methods:

Worldwide the industrial sector is growing to meet the demands of a growing human population. These demands ultimately place stress on natural environments that can result in severe adverse impacts on ecosystems. It is not uncommon for industries to utilize or discharge into local water systems (lakes, rivers or streams), leading to no shortage in the number of potential contaminants of concern. One such area of concern is Se, an element that is often associated with organic rich shales, coal seams, phosphate deposits and mineral deposits. On a global scale activities such as coal mining, metal (e.g., copper, uranium) mining, and phosphate mining, petrochemical operations and agriculture have all been associated with increased Se levels in the surrounding environment (Lemly 2007). Interest in Se lies in it being an essential element that has a unique ability to be affected by numerous environmental factors and its fate and environmental effects have challenged researchers and regulatory agencies ability to define clear guidelines. Many details about how Se can interact and behave in aquatic environments have been determined yet in some ways this knowledge also seems to complicate our ability to clearly define thresholds for the protection of impacted water systems.

In the USA and Canada numerous sites have been identified with elevated levels of Se in watersheds located near various anthropogenic activities. In Canada, Se has been identified as a element of concern in uranium mining and milling effluents discharged in Northern Saskatchewan as well as in coal related activities in Alberta and British Columbia. In the USA two of the earliest and most notable cases were Belews Lake, North Carolina and Martin Reservoir, Texas, both associated with coal-related activities. Also of important note is Kesterson Reservoir in California where aquatic birds were greatly impacted following Se inputs from agricultural sources. Some of the more recent concerns having been associated with phosphate mining activities, such as in the Blackfoot River Watershed in Idaho, USA. A table of highlighted North American cases from the literature provides additional details on concentrations and observed effects (Table 3.1). Concerns related to Se are not restricted to North America, as Australia, Ecuador, United Kingdom, Sweden, Poland, France, Egypt, South Africa, Israel, Russia, India, Hong Kong, and Japan are all places where Se pollution has occurred in fish and other wildlife populations (Lemly 2004). The results of these studies have contributed to the understanding that Se has the potential to bioaccumulate in aquatic systems to

a level that can lead to the "silent" or unnoticed reproductive failure of fish communities as the adult population appears healthy while juvenile recruitment is reduced. Much of the elevated Se is transferred through dietary exposure from the lower trophic levels up to the fish as well as aquatic birds.

Much of the work to date on Se falls into the two broad categories of laboratory experiments or field surveys. One of the greatest advantages of modular mesocosms systems is that they are a great tool to evaluate effects from a field-based perspective while still having the control that is desired in laboratory work. The mesocosm approach is also unique in Se research as most the data dealing with reproduction involves the collection of eggs from mature fish in the field, which are then incubated in the laboratory and evaluated for deformities and survival (McDonald and Chapman 2009). The mesocosm work to date by Hruska and Dubé (2004), Dubé et al. (2005; 2006), Rickwood et al. (2006a,b,c; 2008), Rozon-Ramilo et al. (2011), and Driessnack et al. (2011a,b), have permitted the assessment of reproductive endpoints, beyond survival and deformities, in offspring that were maintained in the same conditions as the adults. The details of one such study are given in much greater detail by Driessnack et al. (2011a, Chapter 2). As presented in the results of that manuscript the data collected in a field based mesocosm study at the Key Lake uranium milling site in Saskatchewan, Canada were extensive and allowed for various analytical methods to be employed in terms of chemistry (total metals, Se speciation) as well as statistical methods such as correlation and Principal Component Analysis (PCA).

It should be noted that correlation is intended to indicate the degree of association between two variables as indicated by the correlation coefficient or Spearman's rho, which can then be tested for a statistically significant association. Another fact of note is that cause/effect relationships are not identified by correlation analysis, identification of those relationships are a component of linear regression. Linear regression is used to predict via a linear regression equation based on a postulated causal relationship, where r^2 provides a measure of the predictive strength of the relationship from the observed data. To evaluate the correlations within the mesocosm data the software program SPSS 18.0 was employed (SPSS, Chicago, IL, USA). Principal Components Analysis was conducted using SPSS 18.0 and was used to evaluate Se in environmental matrices (water and algae), fish tissue (female muscle and ovary, eggs and larvae)

and key observed biological effects (egg size, reproduction). There was also examination of all metals, other then Se, that were measured in the same tissues and environment matrices as above. PCA is useful as it allows variables to be grouped based on the similarity of responses, where the first principal component accounts for as much variability as possible with subsequent components accounting for the remaining portions of the total variance. PCA can also be viewed as a multivariate technique based on the ordination of samples. Analysis facilitates the clustering or classification of samples into groups that are similar. These relationships can be viewed in 2 to 3 dimensions usually, where the distance between points in the mapping reflects the relative dissimilarity of samples. Using this technique we first visualized the relationships between selected biological endpoints (% deformed, hatching success, percent fertilization, percent larval survival, egg size, mean spawns, mean e/f/d and mean normal larvae). A second PCA was carried out to examine the concentration of all metals measured in female muscle, ovaries, eggs and larvae as well as water and sediment. The goals of this second PCA were to identify any additional patterns in treatment effects for the biological endpoints and then to investigate the trends in metal loading in the different environmental and tissue samples.

To begin the analysis the assumptions of normality and homoscedasticity, were tested prior to analyzing for correlation. Failure to meet those assumptions resulted in data being transformed ($\log_{10}$+1 or arcsin) and re-evaluated. If assumptions were still not meet the non-parametric equivalent, the Spearman Rank correlation, was used. It was also decided to generate coefficients of determination or r^2 while graphing the different relationships (e.g. Se in ovary and Se in eggs) to allow for increased comparisons with not only r but also r^2 values reported in the literature.

Previous studies in the USA and Canada have evaluated the correlation and regression between different environmental and fish tissue matrices. The work by Driessnack et al. (2011a) has the added benefit of correlation with reproductive endpoints beyond deformities and survival with multiple fish tissues and the environment. The decision to not use full linear regression models to generate predictive relationships was made based on the fact that multiple factors needed to be addressed and included (e.g. pH, sulfate and reduction potential) to accurately develop a linear regression model that could be considered predictive of Se in different compartments (Simmons and Wallschläger 2005). The primary goal of the present work was to

compare correlationvalues reported in the literature and to evaluate the presence of trends to be later considered for more detailed modeling or further experimental work on identified information gaps.

3.2.1 Selenium Correlation Analysis Results:

Correlation was done between only two points at a time using various combinations from the mesocosm data. From that multiple significant correlations were found during the analysis of the data using Spearman Ranks tests. Significant positive correlations ($p \leq 0.05$) were noted between Se in the water, algae, muscle, ovaries, eggs, larvae and percent (%) deformed larvae. In addition, multiple significant negative correlations ($p \leq 0.05$) were detected between the levels of Se in the environmental and tissue matrices with egg size and larval survival. The results were separated into three phases for ease of analysis: correlations between the environment and adult tissues (Table 3.1), correlations between adult tissues and offspring with egg size and larval deformities (Table 3.2), and then correlations between the biological endpoints (Table 3.3). Additionally Table 3.4 is a combination of all possible correlations and indicates additional associations that allow comparison to studies reported in the literature. Additionally, a Bonferroni correction was done by the software during analysis to avoid Type I error as a result of multiple comparisons.

Between the environmental compartments there was a strong and significant correlation between Se in water and algae (Spearman rho 0.927, $p < 0.001$, and $r^2 = 0.996$) (Table 3.1, Figure 3.1). This supports the contention that the algae/biofilm component is the main route of Se assimilation from the aqueous environment, as the two are strongly related in a linear relationship (Figure 3.1). When examining transfer from water to female fish muscle and ovaries, significant correlations were noted (rho = 0.699 and 0.688, respectively), in addition to moderately strong coefficients of determination (r^2= 0.498 and 0.487, respectively). For algae slightly, stronger correlations were observed between muscle and ovary (rho= 0.752 and 0.729, respectively), while regression analysis generated r^2 values of 0.532 and 0.462 respectively.

Examination of correlations among adult fish tissues indicated a strong correlation between muscle and ovary (rho = 0.865, $p < 0.001$, and $r^2 = 0.821$) (Table 3.2, Figure 3.2a). Examination between concentrations in muscle and eggs and larvae showed strong and

significant correlations (Table 3.2) with moderately strong coefficients of determination, (r^2 = 0.500 and 0.516, respectively) (Figure 3.2b). Ovary Se levels correlated again strongly and significantly to eggs and larvae, but the coefficients of determinations between ovary and egg were moderate, (r^2 = 0.444) while it was much stronger for larvae (r^2 = 0.721) (Table 3.2, Figure 3.2c,d). Evaluation of Se levels between eggs and larvae resulted in a significant association (rho = 0.846, p= 0.001, and r^2 = 0.734) (Table 3.4, Figure 3.2e). Egg size and Se in the ovary was strongly and negatively correlated (rho = -0.763, p = 0.004, and r^2 = 0.763) (Figure 3.3a). In Figure 3.3b again egg size is considered against egg Se levels, similarly to ovary Se there was a strong negative correlation, (rho = -0.848, $p < 0.001$ and r^2 = 0.675). Comparisons between % larval deformities and Se in the eggs was strongly and significantly correlated (rho = 0.809, p =0.001 and r^2 = 0.353) (Figure 3.4a). Further examination of Se in larvae yielded strong correlations with % larval deformities (positive) and % larval survival (negative) with rho = 0.822 and -0.607 ($p < 0.05$) respectively, as well as coefficients of determination of r^2 = 0.574 and 0.275, respectively. Egg Se levels were also strongly and negatively correlated with % survival (rho = -0.736, p = 0.006, r^2 = 0.328) (Figure 3.4 b,c,d)

Additional biological effects were assessed for comparison between egg size, % survival and % deformities, where a significant positive correlation was noted between survival and egg size (rho= 0.628, p = 0.029, r^2 = 0.557) and a significant negative correlation with % larval deformities (rho= -0.770, p=0.003, r^2= 0.490) in that as egg size increased so did survival while the incidence of deformities decreased (Table 3.3, Figure 3.5 a,b). The number of spawns and number of eggs/female/day (e/f/d) were also evaluated to expand the scope of reproductive endpoints for consideration. Again significant ($p < 0.05$) and strong correlations were noted between spawns and e/f/d when compared with egg size and % larval survival (negative relationships) and % larval deformities (positive relationship) (Table 3.3, Figure 3.6, Figure 3.7d, Figure 3.8d). Strong correlation coefficients were also noted for these endpoints. It was also noted that there were no significant correlations between time to hatch or mean number of normal larvae and any of the matrices or other biological effects (data given in Appendix B). Comparison between spawns and e/f/d was included in the expanded correlation table (Table 3.4). The results here indicated stronger relationships in terms of coefficients of determination with the fish tissues for Se (muscle, ovary, egg and larvae) and are depicted in Figures 3.7 and 3.8 (a-c).

Table 3.1: Spearman Rank Correlation Coefficients for comparisons of selenium levels in the environmental matrices and adult tissues. A p-value of less than or equal to 0.05 is considered to be significant, n = 12.

		Se Water	Se Algae	Se Muscle	Se Ovary
Se Water	Spearman's rho	1			
	p-value	-			
Se Algae	Spearman's rho	0.902	1		
	p-value	< 0.001	-		
Se Muscle	Spearman's rho	0.666	0.752	1	
	p-value	0.018	0.005	-	
Se Ovary	Spearman's rho	0.614	0.729	0.865	1
	p-value	0.034	0.007	< 0.001	-

Table 3.2: Spearman Rank Correlation Coefficients for comparisons of selenium levels in the adult and egg/larvae tissues and whole body. A p-value of less than or equal to 0.05 is considered to be significant, n = 12.

		Se Muscle	Se Ovary	Se Egg	Se Larvae	Whole Body Se
Se Muscle	Spearman's rho	1				
	p-value	-				
Se Ovary	Spearman's rho	0.865	1			
	p-value	< 0.001	-			
Se Egg	Spearman's rho	0.836	0.768	1		
	p-value	0.001	0.004	-		
Se Larvae	Spearman's rho	0.717	0.870	0.846	1	
	p-value	0.009	< 0.001	0.001	-	
Whole Body Se	Spearman's rho	0.865	1.000	0.768	0.870	1
	p-value	< 0.001	< 0.001	0.004	<0.001	-

Table 3.3: Spearman Rank Correlation Coefficients for comparisons of biological effects. A p-value of less than or equal to 0.05 is considered to be significant, n = 12.

		Egg Size	% Larval Deform	% Larval Survival	% Hatch	Spawns	E/F/D
Egg Size	Spearman's rho	1					
	p-value	-					
% Larval Deform	Spearman's rho	-0.781	1				
	p-value	0.003	-				
% Larval Survival	Spearman's rho	0.643	-0.611	1			
	p-value	0.024	0.035	-			
% Hatch	Spearman's rho	0.572	-0.576	0.725	1		
	p-value	0.052	0.050	0.008	-		
Spawns	Spearman's rho	-0.768	0.656	-0.576	-0.509	1	
	p-value	0.004	0.021	0.50	0.091	-	
E/F/D	Spearman's rho	-0.777	0.775	-0.699	-0.585	0.760	1
	p-value	0.003	0.003	0.011	0.046	0.004	-

% Larval Deform – percent larval deformities, % Larval Survival – percent larval survival to 5-days post hatch, % Hatch – percent hatching success, Spawns- number of spawning events, E/F/D – eggs/female/day

Table 3.4: Spearman Rank Correlation Coefficients for comparisons of selenium (Se) levels in the adult and egg/larvae tissues and egg size and biological effects. A p-value of less than or equal to 0.05 is considered to be significant.

		Se Water	Se Algae	Se Muscle	Se Ovary	Se Egg	Se Larvae	Whole Body Se	Egg Size	%Larval Deform	% Larval Survival	% Hatch	Spawns	E/F/D
Se Water	rho	1												
	p-value	-												
Se Algae	rho	0.902	1											
	p-value	< 0.001	-											
Se Muscle	rho	0.666	0.752	1										
	p-value	0.018	0.005	-										
Se Ovary	rho	0.614	0.729	0.865	1									
	p-value	0.034	0.007	< 0.001	-									
Se Egg	rho	0.737	0.755	0.836	0.768	1								
	p-value	0.006	0.005	0.001	0.004	-								

Table 3.4 Continued														
Se Larvae	rho	0.753	0.756	0.717	0.870	0.846	1							
	p-value	0.005	0.004	0.009	< 0.001	0.001	-							
Whole Body Se	rho	0.614	0.729	0.865	1.000	0.768	0.870	1						
	p-value	0.034	0.007	< 0.001	< 0.001	0.004	<0.001	-						
Egg Size	rho	-0.751	-0.799	-0.871	-0.768	-0.844	-0.809	-0.768	1					
	p-value	0.005	0.002	< 0.001	0.004	< 0.001	0.001	<.004	-					
% Larval Deform	rho	0.739	0.715	0.786	0.768	0.810	0.824	0.768	-0.781	1				
	p-value	0.006	0.009	0.002	0.004	0.001	0.001	0.004	0.003	-				
% Larval Survival	rho	-0.708	-0.829	-0.785	-0.715	-0.736	-0.607	-0.715	0.643	-0.611	1			
	p-value	0.10	0.001	0.003	0.009	0.006	0.037	0.009	0.024	0.035	-			

Table 3.4 Continued

% Hatch	rho	-0.644	-0.702	-0.642	-0.635	-0.514	-0.620	-0.635	0.572	-0.576	0.725	1	
	p-value	0.024	0.011	0.024	0.026	0.088	0.031	0.026	0.052	0.050	0.008	-	
Spawns	rho	0.460	0.546	0.701	0.833	0.768	0.854	0.833	-0.768	0.656	-0.576	-0.509	1	
	p-value	0.133	0.066	0.011	0.001	0.004	<	0.001	0.004	0.021	0.50	0.091	-	
E/F/D	rho	0.689	0.788	0.732	0.718	0.757	0.785	0.718	-0.777	0.775	-0.699	-0.585	0.760	1
	p-value	0.013	0.002	0.007	0.009	0.004	0.003	0.009	0.003	0.003	0.011	0.046	0.004	-

% Larval Deform – percent larval deformities

% Larval Survival – percent larval survival to 5-days post hatch

% Hatch – percent hatching success

Spawns- number of spawning events

E/F/D – eggs/female/day

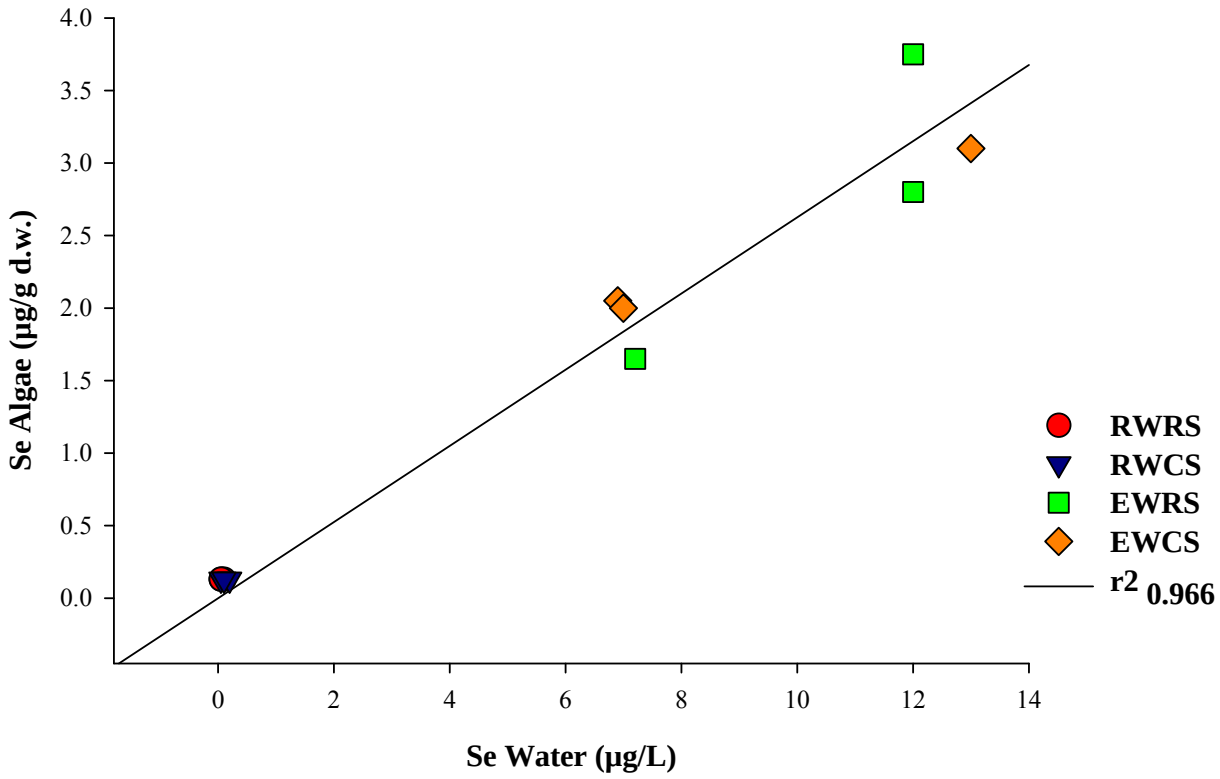

Figure 3.1: Scatter plot for Se in the water (µg/L) and algae in µg/g dry weight (d.w.) with an r^2 value of 0.966 calculated from linear regression, and n= 12.

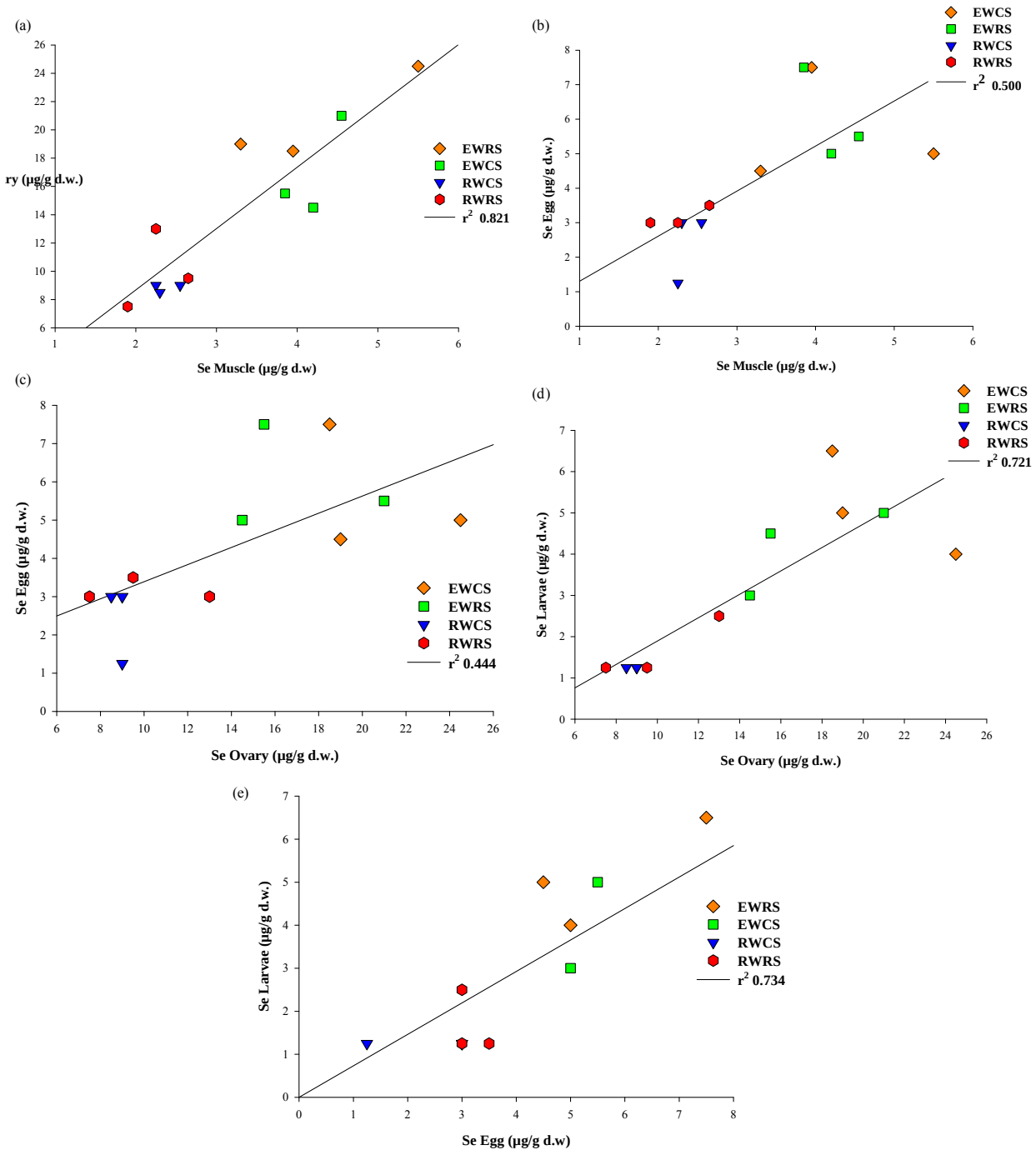

Figure 3.2: Scatter plot for selenium (Se) in female and offspring tissues given as µg/g dry weight (d.w) with coefficient of determination (r^2) given in the legend, and n= 12. (a) Se in ovary and muscle (b) Se in egg and muscle (c) Se in egg and ovary (d) Se in larvae and ovary and (e) Se in larvae and egg.

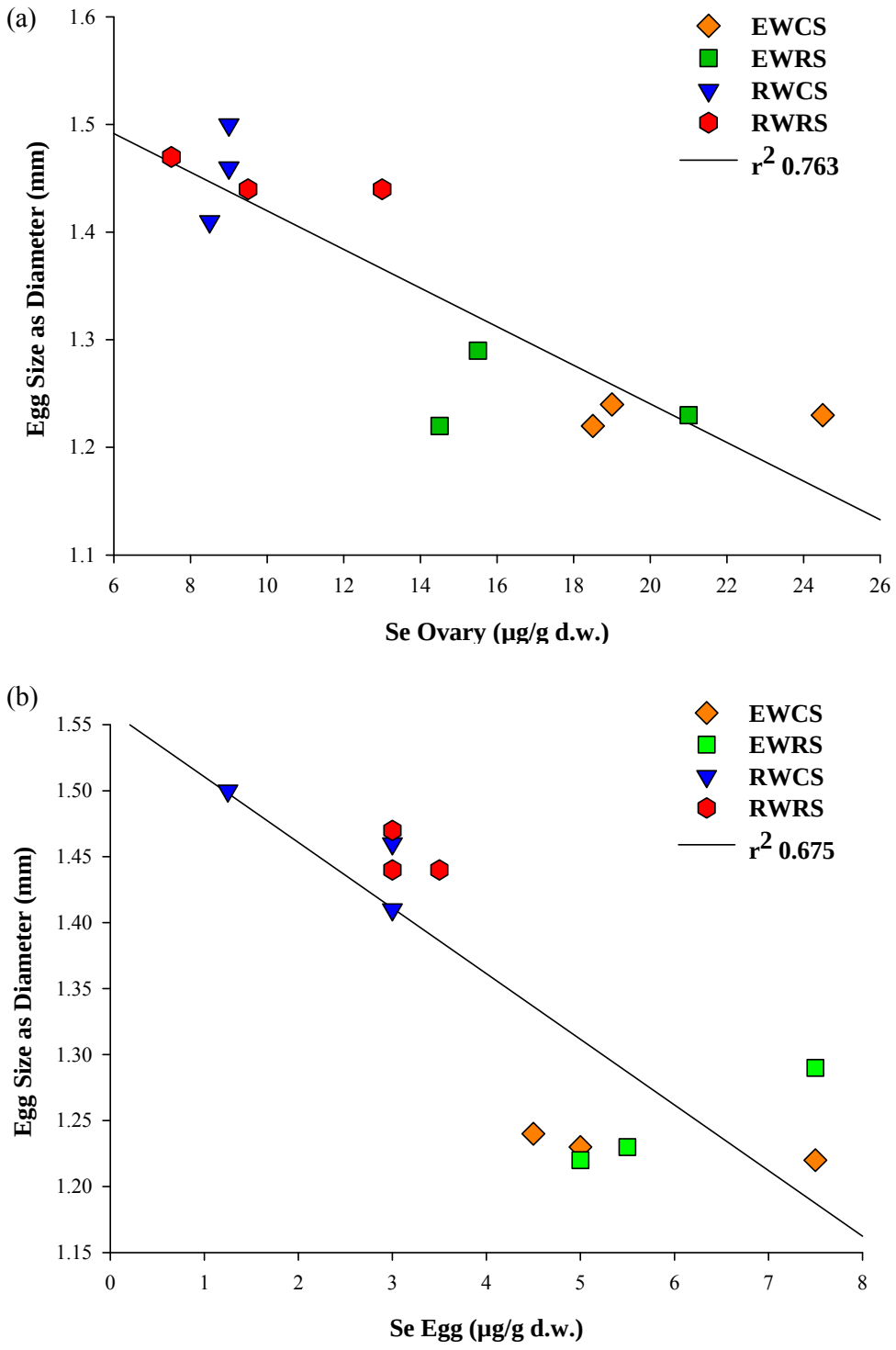

Figure 3.3: Scatter plot for selenium (Se) in egg and ovary given as µg/g dry weight (d.w) and egg size as diameter in millimeters (mm) with coefficient of determination (r^2) given in the legend, and n= 12. (a) Se in egg and egg size (b) Se in ovary and egg size.

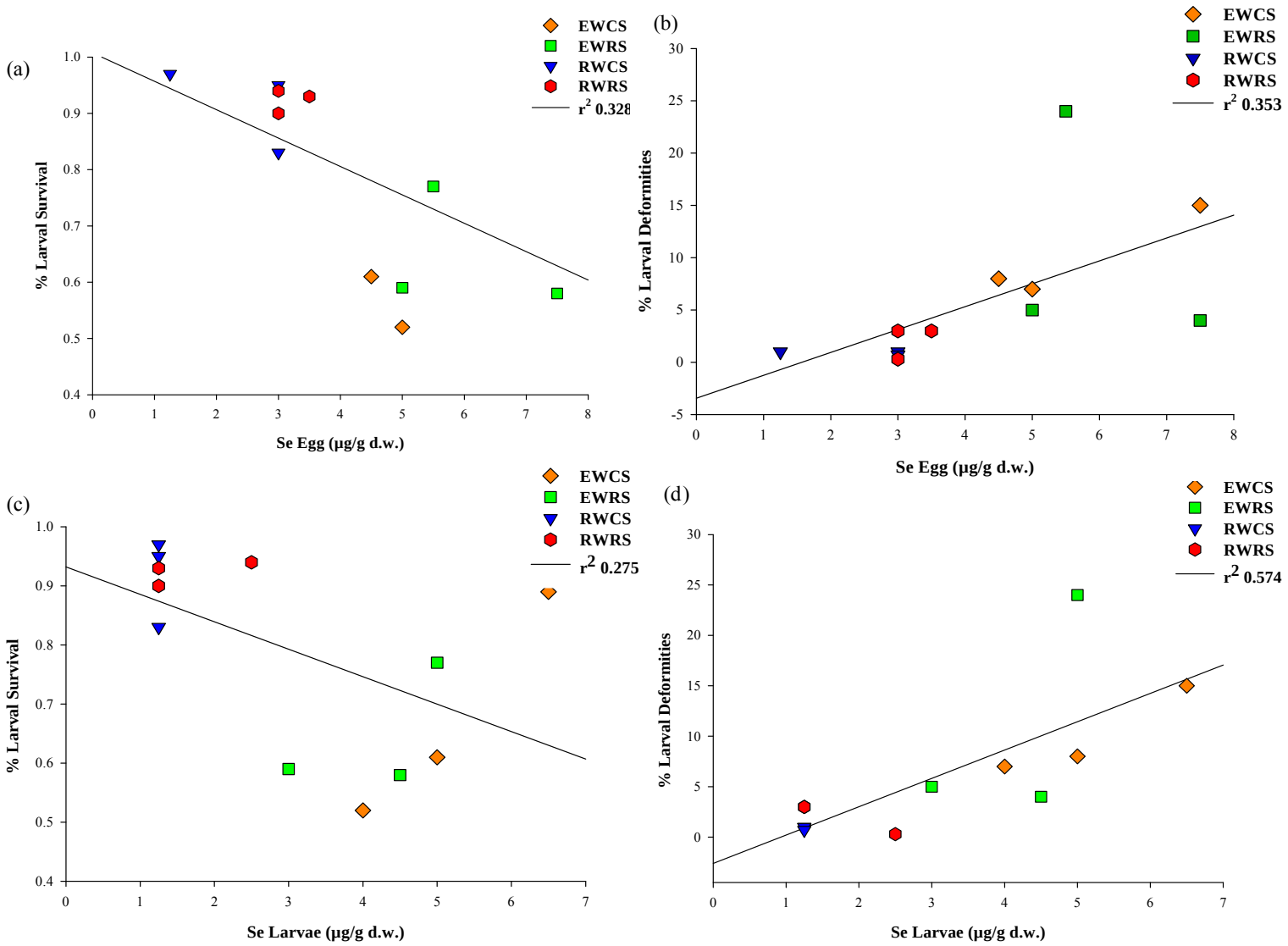

Figure 3.4: Scatter plot for selenium (Se) in egg and percent (%) larval survival (a) and % larval deformities (b). Se in larvae and % larval survival (c) and d% larval deformities (d) with coefficient of determination (r^2) given in the legend, and n= 12.

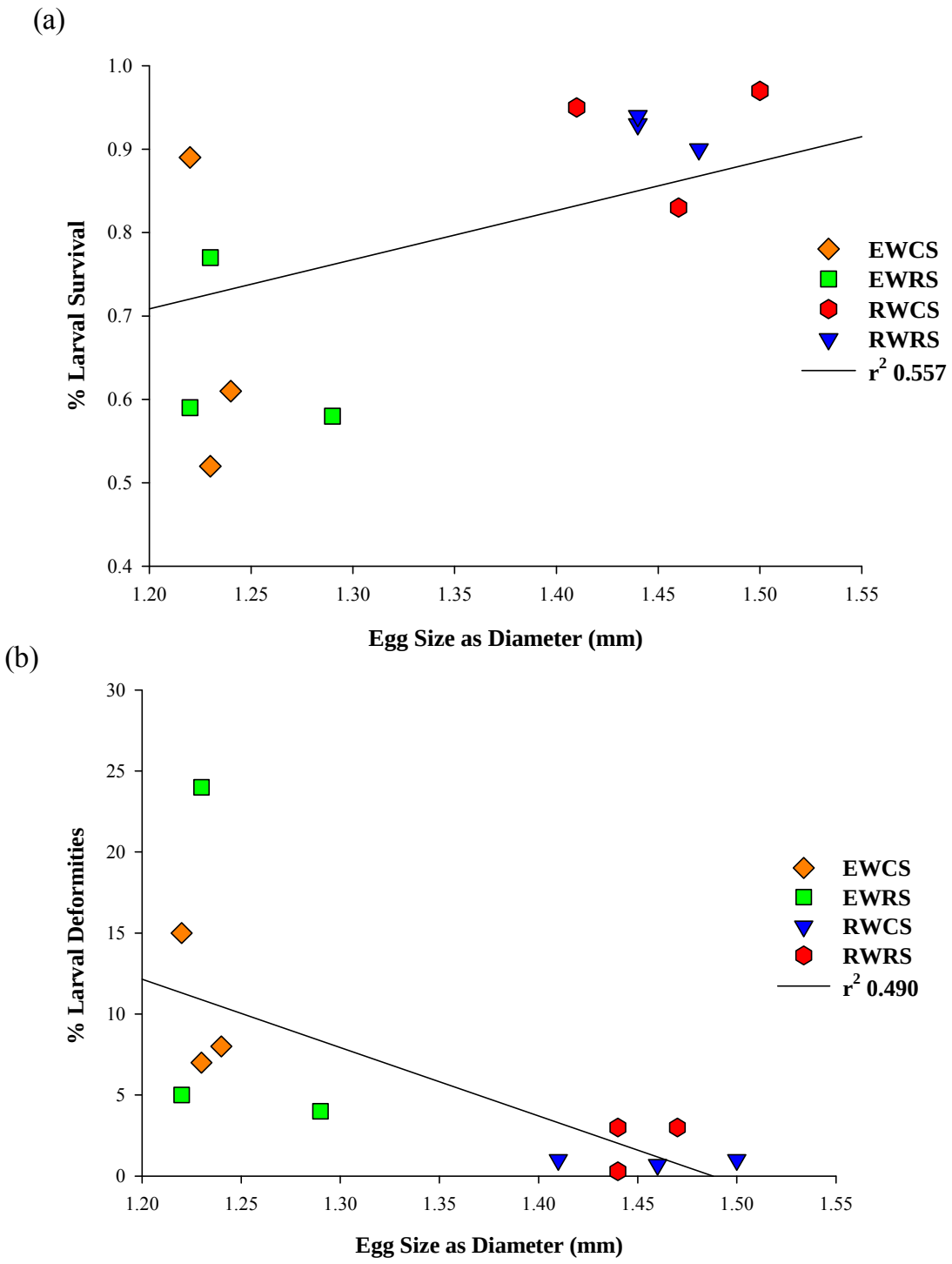

Figure 3.5: Scatter plot of egg size as diameter in millimeters (mm) compared to (a) percent (%) larval survival and (b) % larval deformities with the coefficient of determination (r^2) given in the legend, and n= 12.

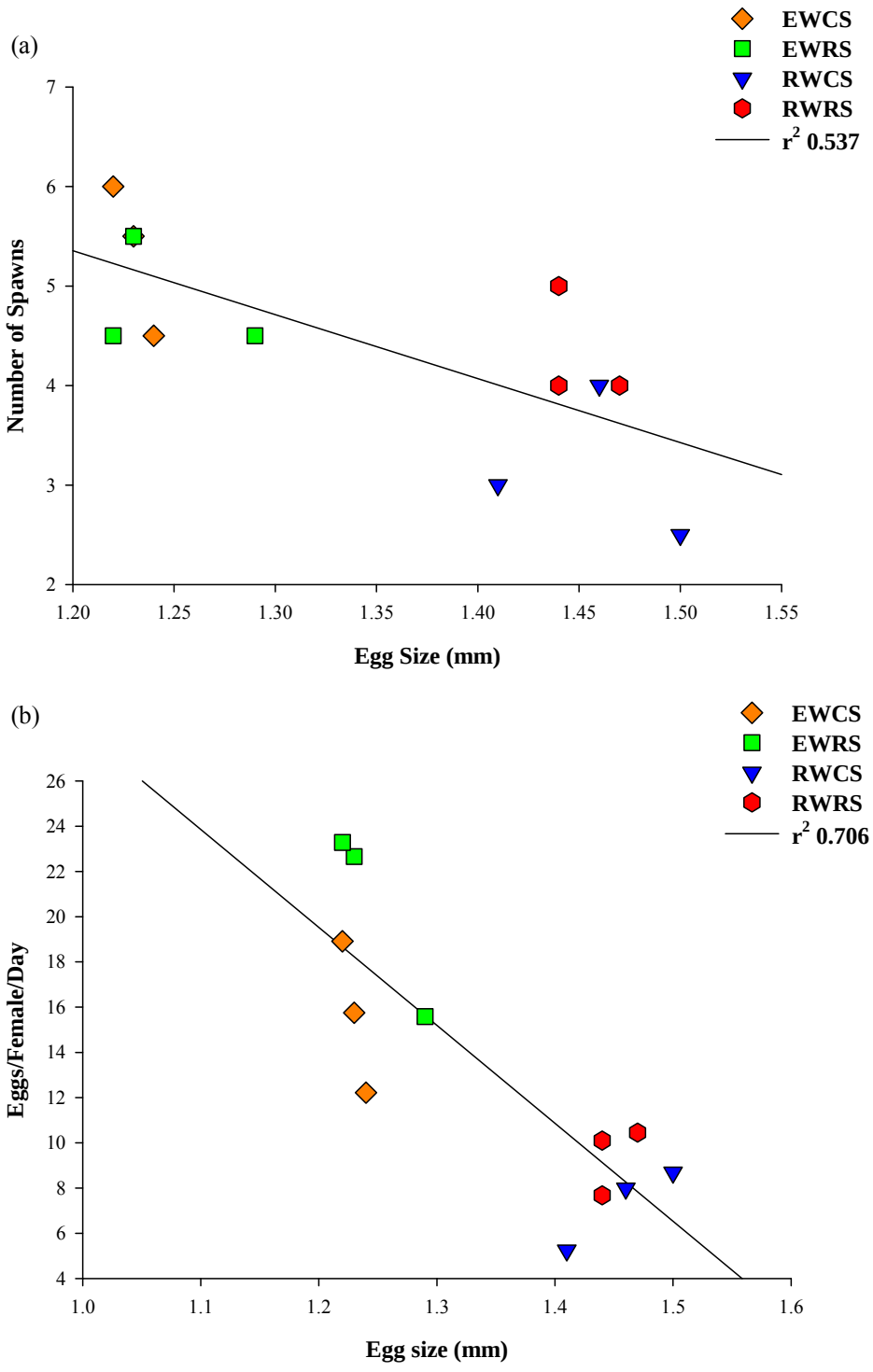

Figure 3.6: Scatter plot of egg size as diameter in millimeters (mm) compared to (a) number of spawns (b) eggs/female/day with the coefficient of determination (r^2) given in the legend, and n= 12.

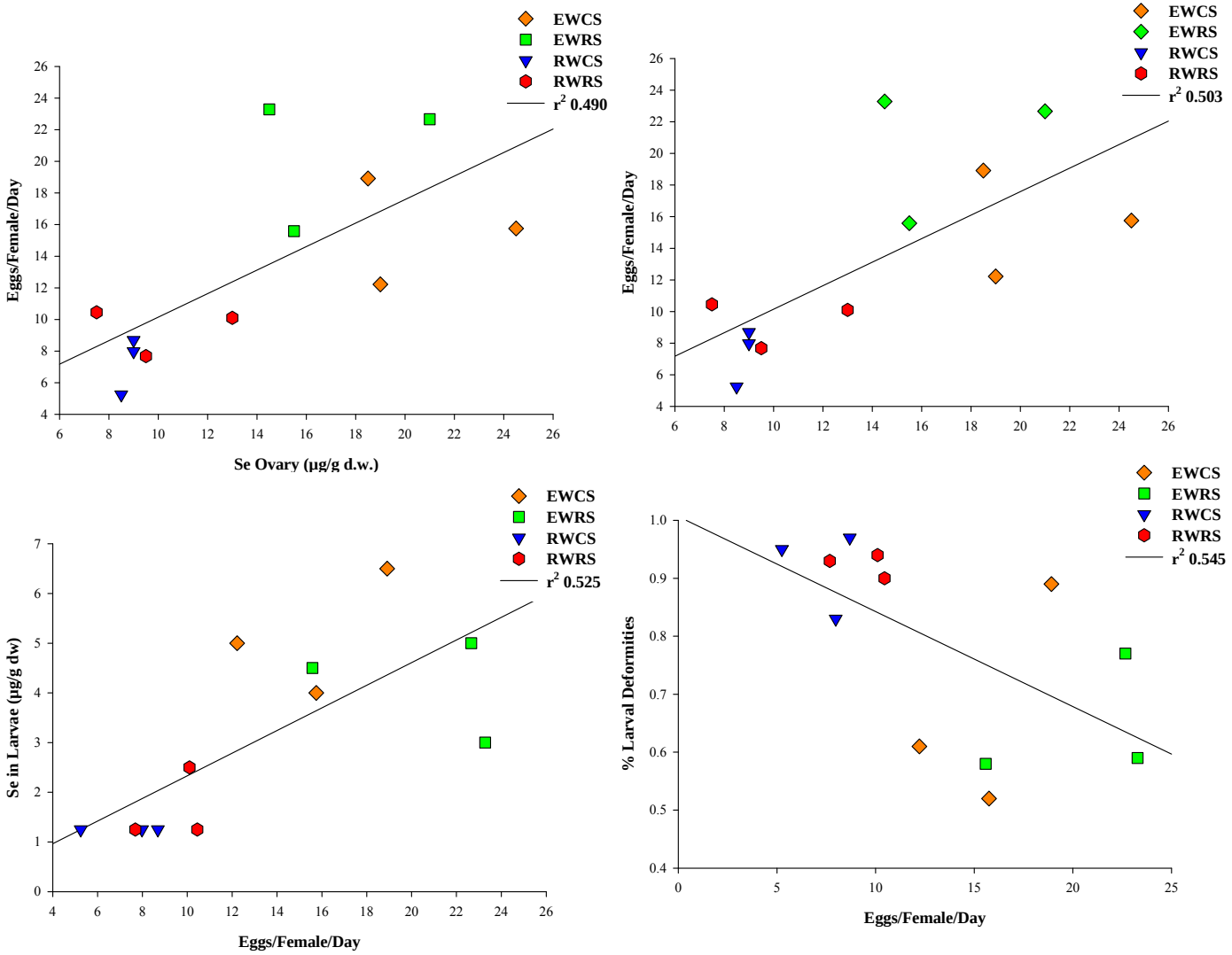

Figure 3.7: Scatter plots of eggs/female/day produced per trio compared to (a) selenium (Se) in ovary (b) Se in eggs (c) Se in larvae (d) percent (%) larval deformities with the coefficient of determination (r^2) indicated in the legend, and n= 12.

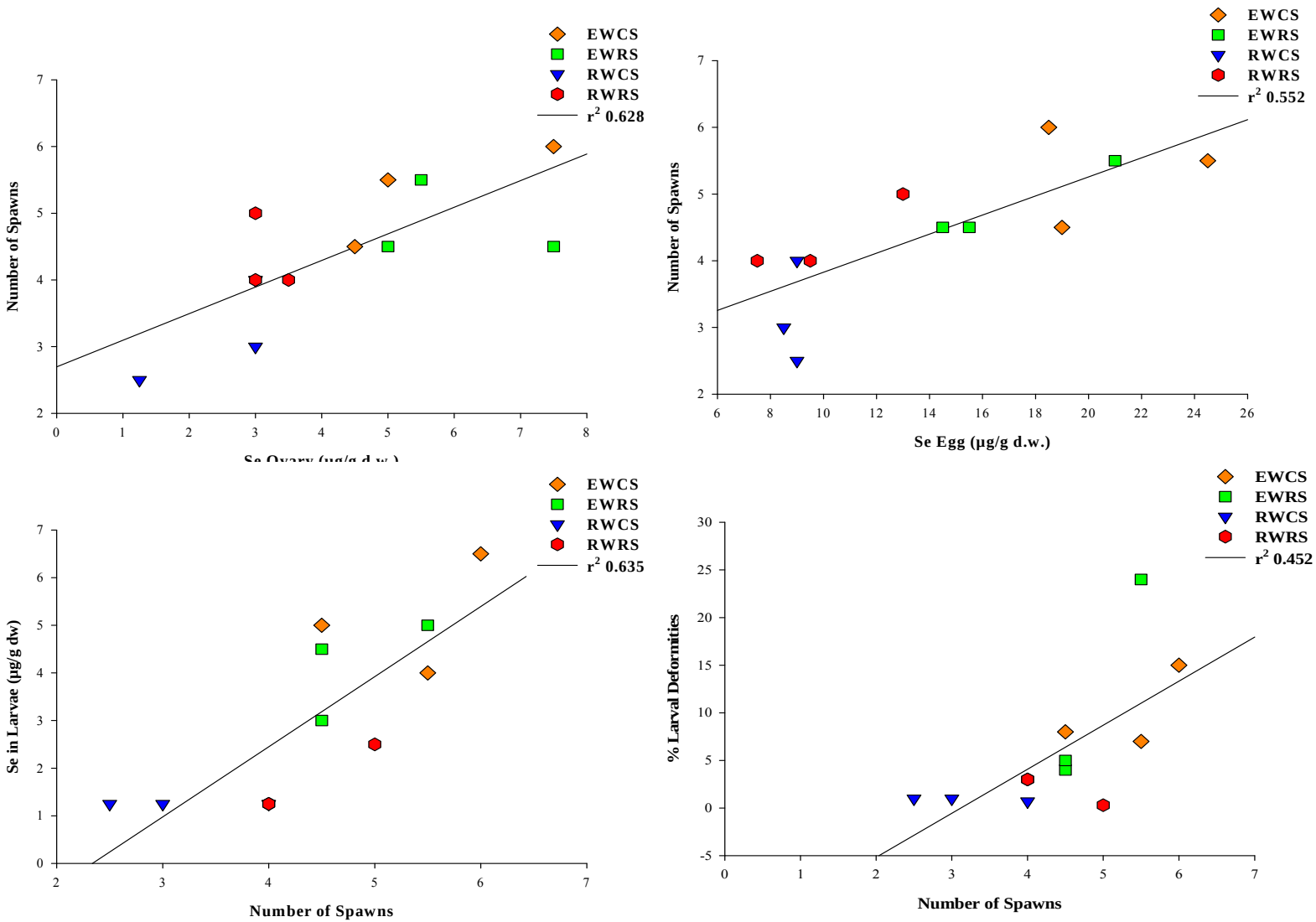

Figure 3.8: Scatter plots of number of spawns per trio of fathead minnows compared to (a) selenium (Se) in ovary (b) Se in eggs (c) Se in larvae (d) percent (%) larval deformities with the coefficient of determination (r^2) indicated in the legend, and n= 12.

3.2.2: Rubidium Correlation Analysis Results:

Additional correlation work was done for the values of Rb in the different matrices as for Se. This was done for two reasons: first that the effluent is a complex mixture and other components should be evaluated other than Se. The second being that work by Driessnack et al. (2011a, Chapter 2) and Rozon-Ramilo et al. (2011) both noted increased levels of Rb in water and fish tissues following exposure to effluents elevated in metals. In addition, limited work has been done in regards to Rb, it has been shown to bioaccumulate in ecosystems, similarly to Se, and that it has been shown to reduce spermatogenesis in eel testicular cell lines (Campbell et al. 2005; Yamaguchi et al. 2007). Therefore the relationship between Rb and fish reproduction was further explored, though other metals could have been considered as well.

The results of the Spearman ranks also showed strong and significant coefficients of determination for Rb. Table 3.5 lists all possible combinations, as was done for Se. Rb in the water correlated most strongly with number of spawns, e/f/d, % larval deformities, and Rb in eggs and larvae all positively as well as negatively with egg size (Table 3.5, Figure 3.9 a,b, Figure 3.10 a,b,c,d) Rb in the water and egg size also had a very strong $r^2 = 0.950$, as well as for Rb in water and e/f/d ($r^2 = 0.733$), Rb in eggs ($r^2 = 0.743$) and Rb in larvae ($r^2 = 0.823$). Rb in algae did not correlate strongly with either Rb in water, fish muscle or ovary tissue (rho < 0.6), however Rb in algae did correlate negatively, strongly and significantly with egg size (rho = -0.719, p = 0.008) (Table 3.5, Figure 3.11). Rb in egg tissue was correlated with Rb in ovary (rho = 0.724, p = 0.039) as well as for egg size (rho = -0.830, p = 0.001) and % larval deformities (rho = 0.815, p = 0.001). Plotting of these correlations indicated an r^2 of 0.664 for egg size and Rb in eggs but a much weaker coefficient for Rb in eggs and Rb in ovary tissues with $r^2 = 0.268$ (Figure 3.12 a,b). In regards to Rb in larvae tissue again a strong, negative and significant correlation was noted for % hatching success (rho = 0.-845, p = 0.001) and once plotted a coefficient of determination of $r^2 = 0.601$ which is moderately strong (Figure 3.13 a). Rb in larvae was also strongly related to egg size (rho = -0.781, p = 0.003) and % larval deformities (rho = 0.755, p = 0.005) (Table 3.5, Figure 3.13 b,c).

Table 3.5: Spearman Rank Correlation Coefficients for comparisons of rubidium (Rb) levels in the adult and egg/larvae tissues and egg size and biological effects. A p-value of less than or equal to 0.05 is considered to be significant, n = 12.

		Rb Water	Rb Algae	Rb Muscle	Rb Ovary	Rb Egg	Rb Larvae	Egg Size	% Larval Deform	% Larval Survival	% Hatch	Spawns	E/F/D
Rb Water	rho	1											
	p-value	-											
Rb Algae	rho	0.596	1										
	p-value	0.041	-										
Rb Muscle	rho	0.744	0.302	1									
	p-value	0.006	0.340	-									
Rb Ovary	rho	0.636	0.394	0.724	1								
	p-value	0.026	0.205	0.008	-								
Rb Egg	rho	0.847	0.720	0.720	0.724	1							
	p-value	0.001	0.008	0.008	0.039	-							

Table 3.5 Continued													
Rb Larvae	rho	0.723	0.638	0.737	0.622	0.716	1						
	p-value	0.008	0.025	0.006	0.031	0.009	-						
Egg Size	rho	-0.856	-0.719	-0.791	-0.599	-0.830	-0.781	1					
	p-value	< 0.001	0.008	0.002	0.040	0.001	0.003	-					
% Larval Deform	rho	0.842	0.695	0.563	0.490	0.815	0.755	-0.781	1				
	p-value	0.001	0.012	0.057	0.106	0.001	0.005	0.003	-				
% Larval Survival	rho	-0.676	-0.630	-0.581	-0.586	-0.563	-0.655	0.643	-0.611	1			
	p-value	0.016	0.001	0.047	0.045	0.056	0.021	0.024	0.035	-			
% Hatch	rho	-0.515	-0.427	-0.581	-0.358	-0.465	-0.845	0.572	-0.576	0.725	1		
	p-value	0.087	0.166	0.047	0.253	0.128	0.001	0.052	0.050	0.008	-		
Spawns	rho	0.893	0.479	0.687	0.608	0.608	0.733	-0.767	0.658	-0.576	-0.490	1	
	p-value	0.000	0.115	0.014	0.036	0.036	0.007	0.004	0.020	0.50	0.106	-	

Table 3.5 Continued

E/F/D	rho	0.876	0.690	0.553	0.474	0.754	0.669	-0.782	0.778	-0.699	-0.573	0.760	1
	p-value	<0.001	0.013	0.062	0.120	0.005	0.017	0.003	0.003	0.011	0.051	0.004	-

% Larval Deform – percent larval deformities

% Larval Survival – percent larval survival to 5-days post hatch

% Hatch – percent hatching success

Spawns- number of spawning events

E/F/D – eggs/female/day

82

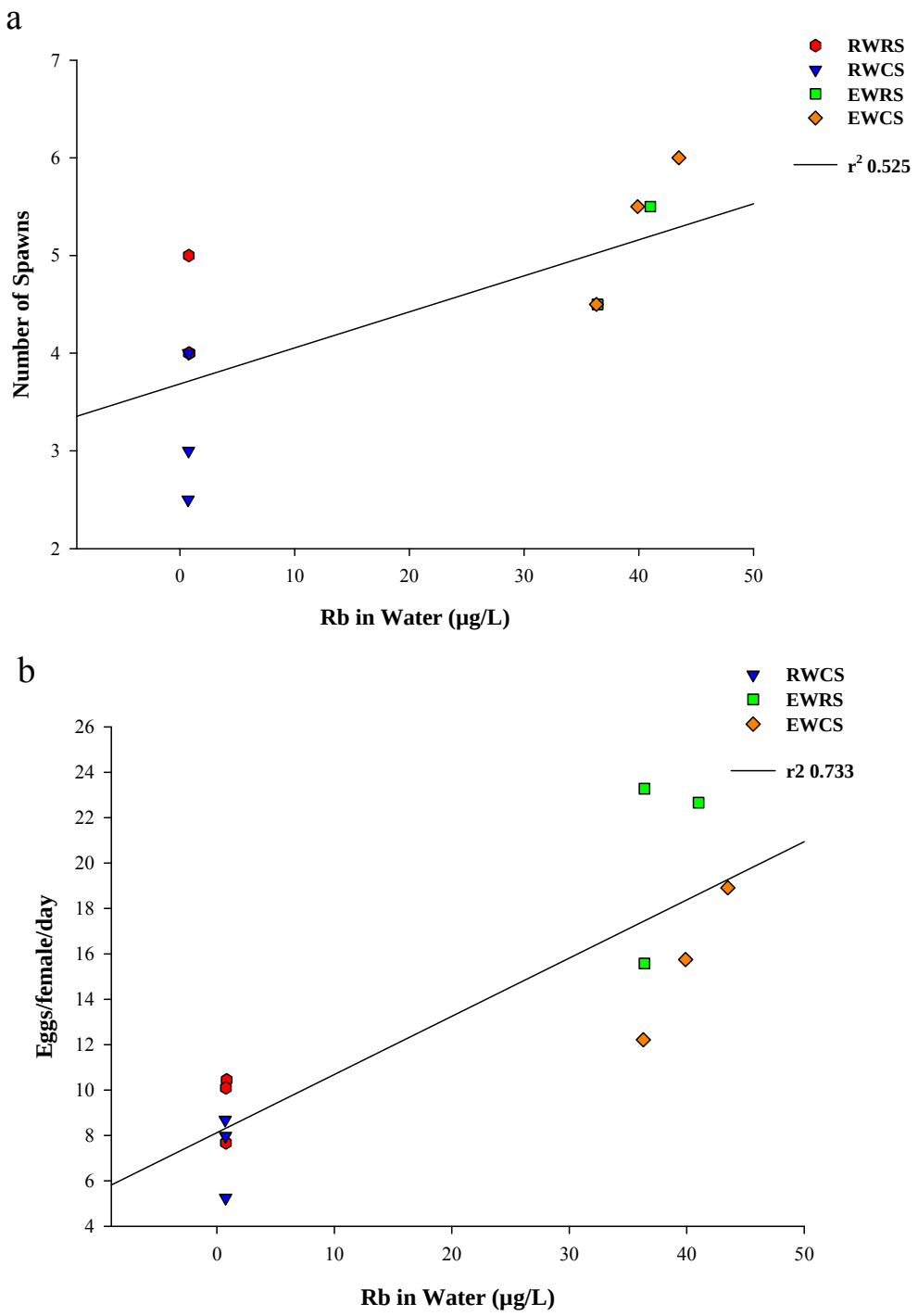

Figure 3.9: Scatter plot of rubidium (Rb) in the water (µg/L) as related to number of spawns (a) and eggs/female/day (b) with the coefficient of determination in the legend, and n= 12.

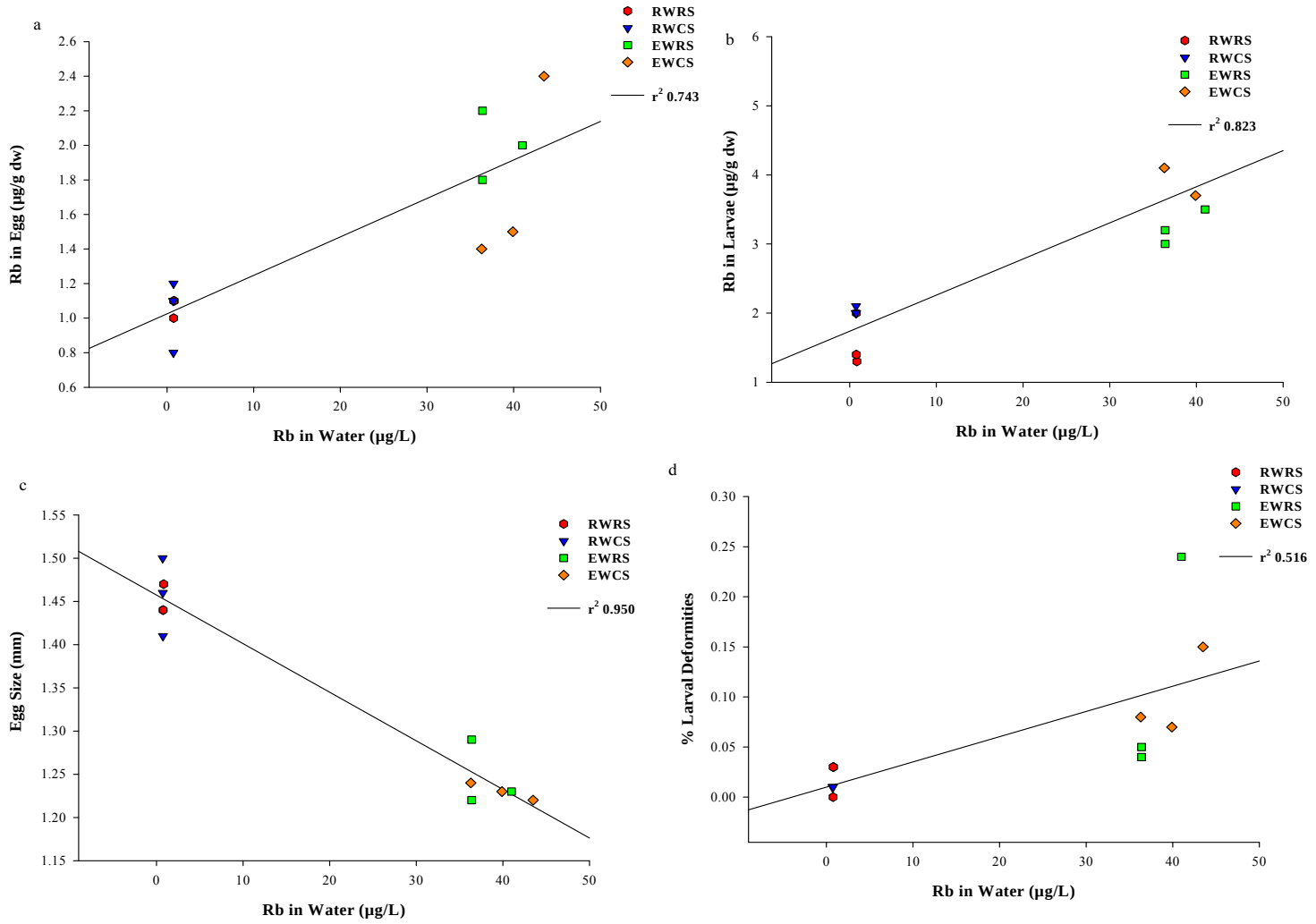

Figure 3.10**:** Scatter plots of rubidium (Rb) in water as related to Rb in eggs (a) and larvae (b). Rb in water as it relates to egg size as diameter (mm) (c) and percent (%) larval deformities (d) with the coefficient of determination in the legend, and n= 12.

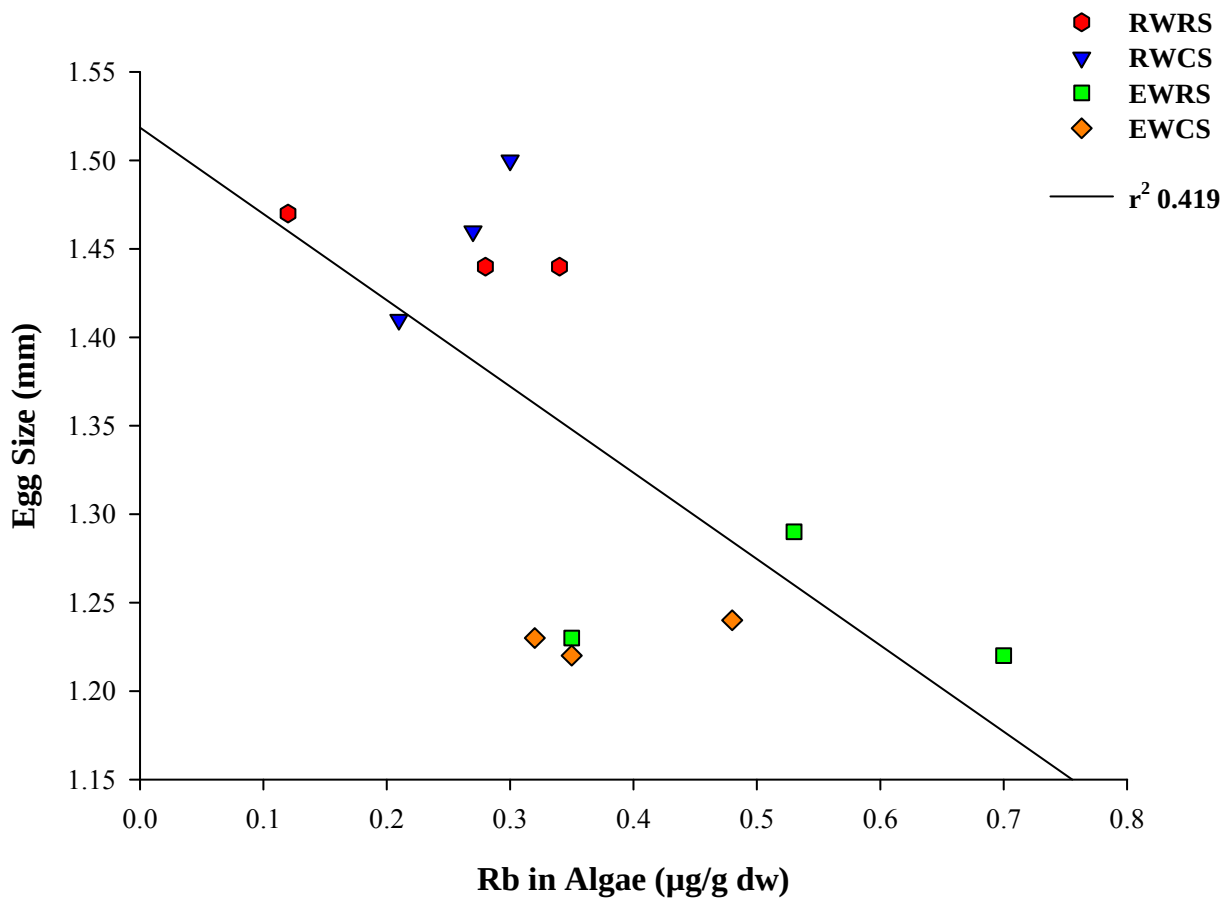

Figure 3.11: Scatter plot of rubidium (Rb) in algae (µg/g dry weight (dw)) and eggs size as diameter (mm) with the coefficient of determination (r^2) indicated in the legend, and n= 12.

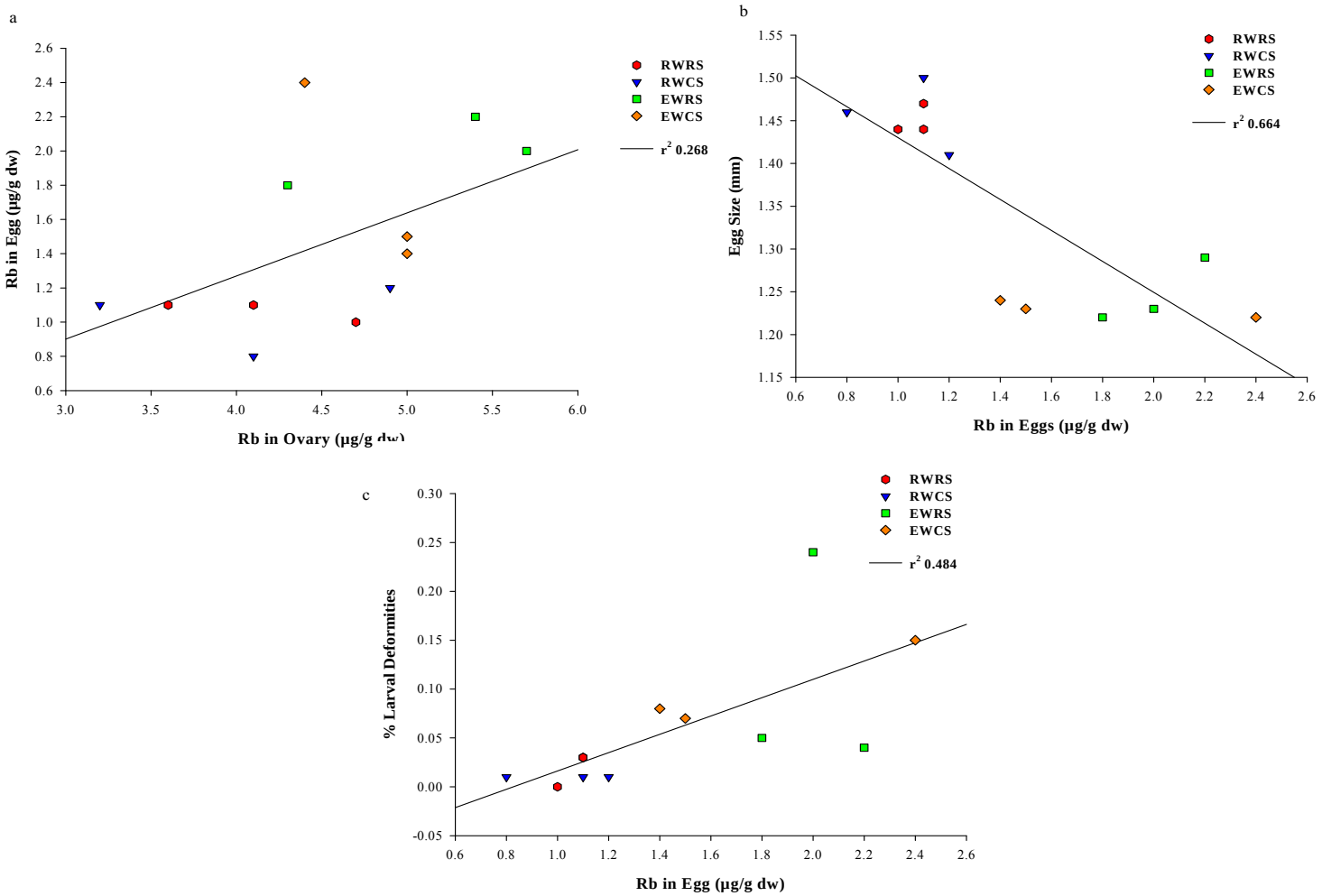

Figure 3.12: Scatter plot of rubidium (Rb) in ovary tissue (µg/g dry weight (dw)) and (a) Rb in egg tissue (µg/g dw). Rb in egg tissue (µg/g dw) and (b) egg size as diameter (mm) and (c) % larval deformities with the coefficient of determination (r^2) indicated in the legend, and n= 12.

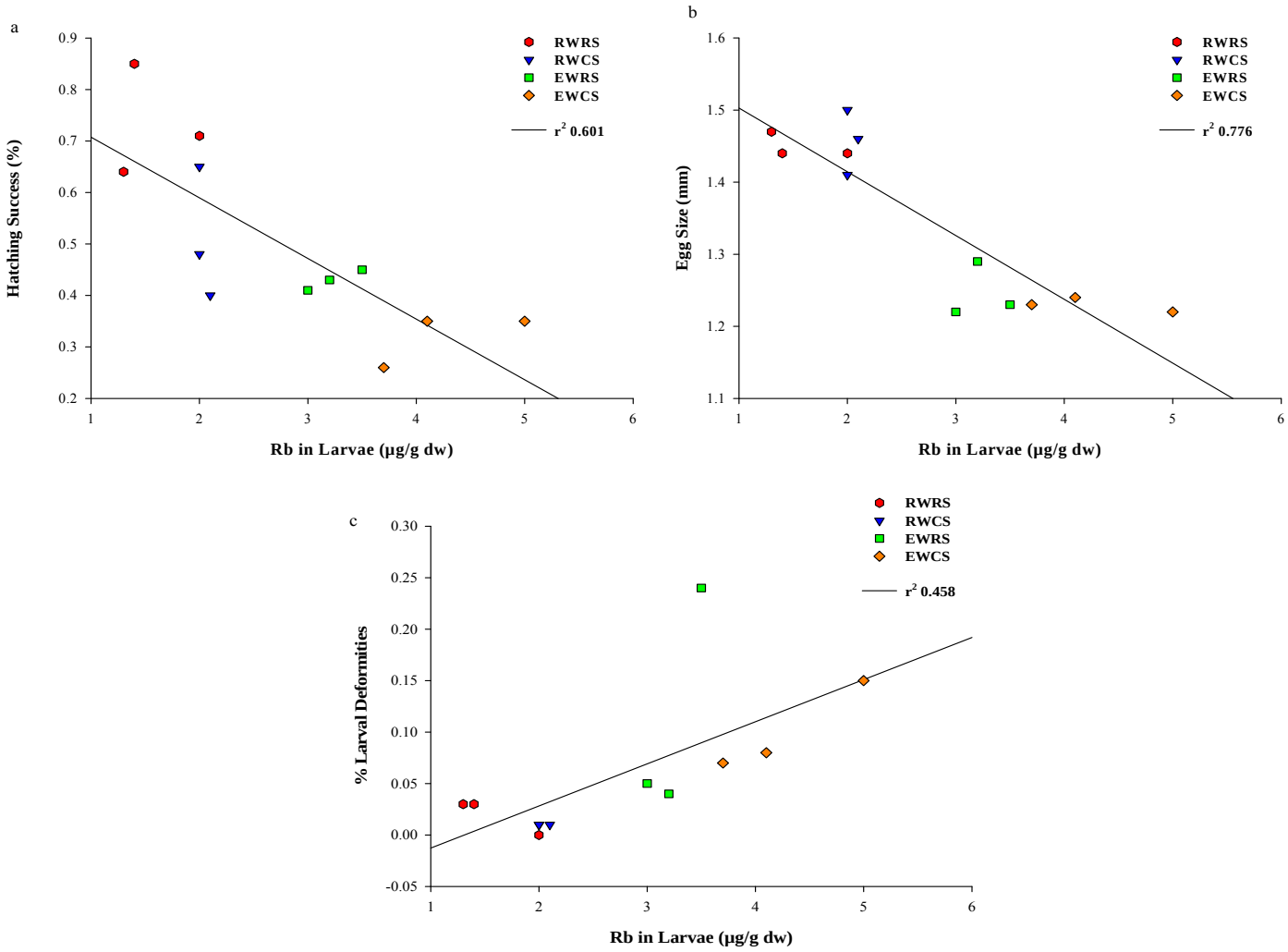

Figure 3.13: Scatter plot of rubidium (Rb) in larvae tissue (µg/g dry weight (dw)) and (a) percent (%) hatching success, (b) egg size as diameter (mm) and (c) % larval deformities with the coefficient of determination (r^2) indicated in the legend, and n= 12.

3.2.3 Principal Component Analysis Results:

In terms of the biological endpoints, results of the PCA continue to support the contention that biological effects result primarily from a water based exposure, where the RW treatments grouped together and EW treatments grouped together. There were no sediment type groupings identified in the ordination plots (Figure 3.14). There was one EWRS sample that was not included in either of the two groupings, but was left in the plots for completeness. Evaluation of the principal component plot (Figure 3.15) indicates groupings of the biological endpoints considered in the PCA and from this it can be seen that % hatching success, larval survival and egg size are similar, while % deformities, e/f/d and number of spawns are another group. The location of normal larvae and % fertilization are not strongly associated with each other or the previously mentioned biological endpoints. When the rotated component matrix was examined, egg size (0.849), % hatching success (0.885), % larval survival (0.878) comprised most of component 1 and % deformities (0.845), fertilization (-0.813) and normal larvae (0.944) comprised most of the variance explained by component 2 (Table 3.6).

When the metals were evaluated across all sample types, variance was compressed into 3 components for ease of visualization. The data was also standardized, by subtracting the variable's sample mean from each value and then dividing the difference by the sample standard deviation, to reduce the potential for over-domination of a metal from the analytical dataset. The component plot based on metals indicates primarily three groupings of metals. Metals loading strongest in each component were as follows; Component 1 Al, Co, Fe, Mn, Mo, Ni, Ti and V; Component 2 Hg, Cd, Cr, and Tl; Component 3 As, Li, Se and Zn (Table 3.7, Figure 3.16). The factor scores were scatter plotted for the 3 components allowing for evaluation of trends related to sample type (Figure 3.17a-c). In Figure 3.17a it can be observed that eggs and larvae are related but separate from the other samples, while ovary and muscle are related and ordinate between water and algae. The algae and water are separate but only to a limited extent as the algae samples are diffuse in their distribution. Figure 3.17b shows a larger separation between the majority of the water and algae data than Figure 3.17a. Muscle and ovary continue to group together, and egg and larvae are related as well. It is noted that there is some relationship between algae and the eggs and larvae also not suggested in Figure 3.17a. The ordination in Figure 3.17c indicates a fairly large distance between fish tissues and the water samples. It also

Table 3.6: Loading of biological effects in PCA for each of the two components. Numbers closet to +/- 1 loaded the strongest in each component.

	Component 1	Component 2
% Deformities	-0.381	0.845
Egg Size	0.849	-0.426
% Hatching	0.885	0.023
% Survival	0.878	-0.035
% Fertilization	0.166	-0.813
Spawn	-0.603	0.497
Eggs/female/day	-0.658	0.662
Normal Larvae	0.141	0.944

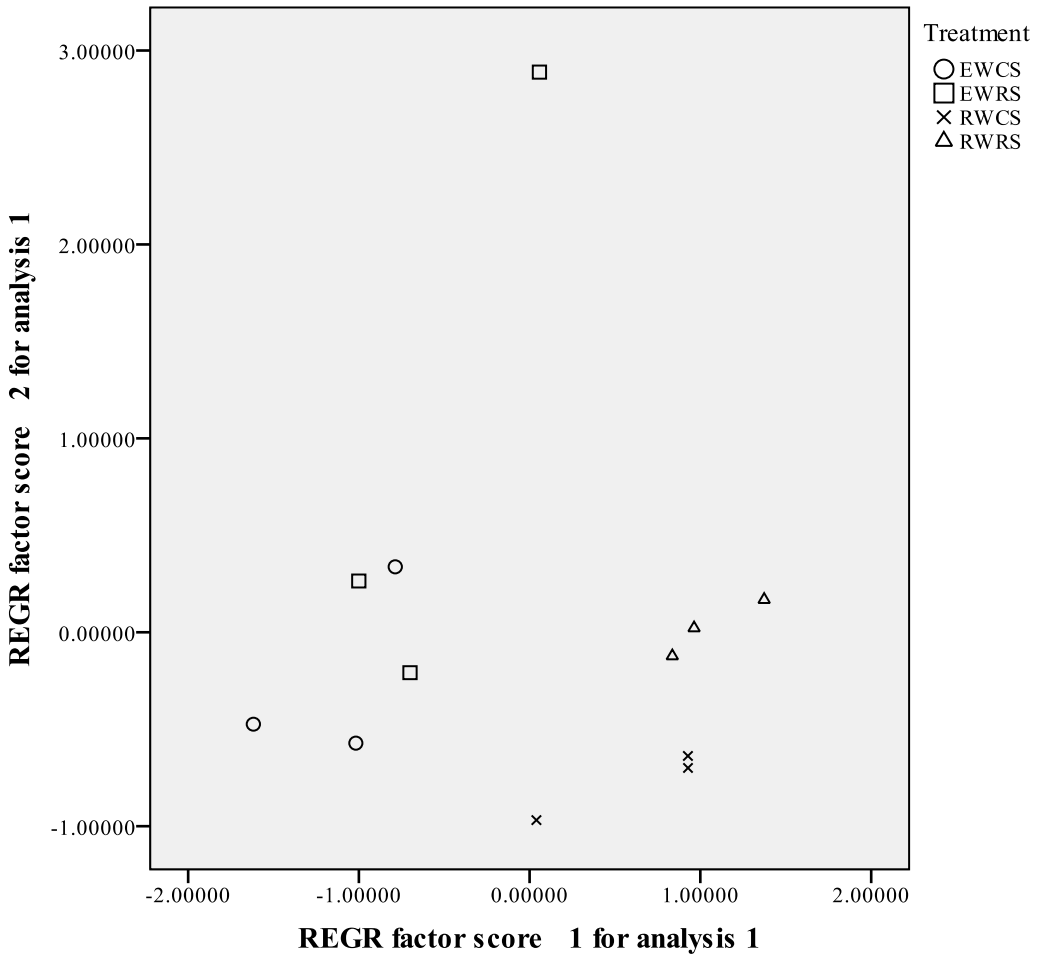

Figure 3.14: Factor score scatter plot based on treatments: Reference water: Reference sediment (RWRS), Reference water: Contaminated sediment (RWCS), 25% Diluted Effluent: Reference sediment (EWRS) and 25% Diluted Effluent: Contaminated sediment (EWCS) following analysis of biological effects endpoints. Where REGR refers to the regression factor score assigned to the raw data during PCA.

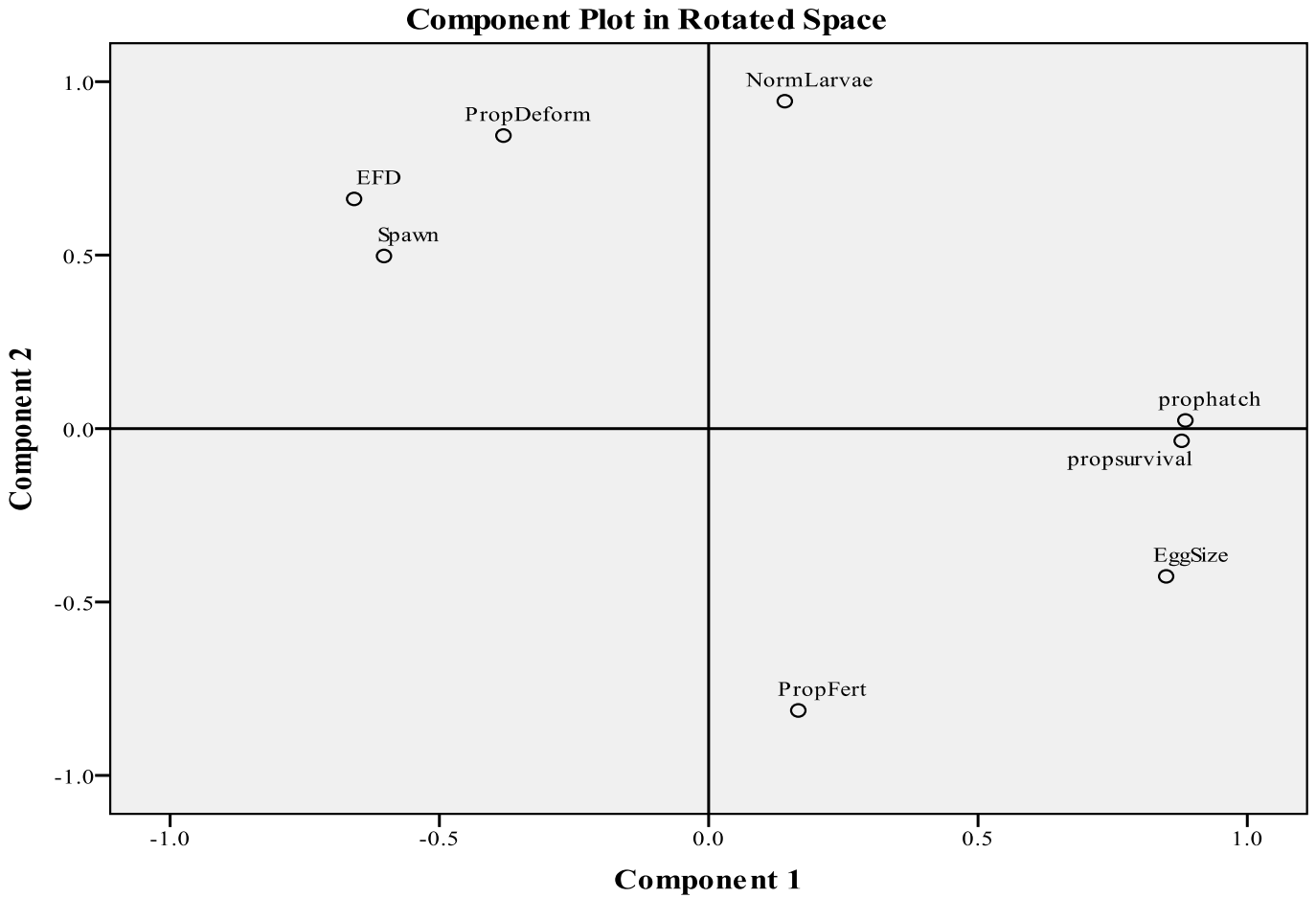

Figure 3.15: PCA component plot indicating the loading or grouping of similar biological effect endpoints, where eggs/female/day (EFD), mean number of normal larvae (NormLarvae), number of spawns (Spawns), percent fertilization (PropFert), percent hatching success (prophatch), percent larval survival (propsurvival), percent larval deformities (PropDeform) and egg size as diameter (EggSize) are represented.

Table 3.7: Loading of metals from the combined total metals PCA for each of the three components. Numbers closest to +/- 1 loaded the strongest in each component.

	Component 1	Component 2	Component 3
Hg	-0.143	0.832	0.035
Al	0.946	-0.044	-0.023
As	-0.083	-0.287	-0.756
Ba	-0.200	-0.414	0.747
Cd	0.002	0.819	0.144
Cr	0.345	0.868	-0.046
Co	0.911	0.027	-0.075
Cu	0.592	-0.042	0.153
Fe	0.864	-0.170	0.123
Pb	0.070	0.522	0.021
Li	-0.210	-0.325	-0.743
Mn	0.918	-0.168	0.006
Mo	0.747	0.418	-0.211
Ni	0.954	0.138	-0.085

Table 3.7 Continued			
Rb	-0.118	-0.162	-0.403
Se	-0.068	-0.045	0.672
Sr	-0.172	-0.180	0.338
Tl	-0.082	0.922	0.002
Sn	-0.067	0.519	-0.006
Ti	0.844	0.055	-0.83
U	0.681	-0.071	-0.409
V	0.858	0.367	-0.060
Zn	-0.222	-0.096	0.805

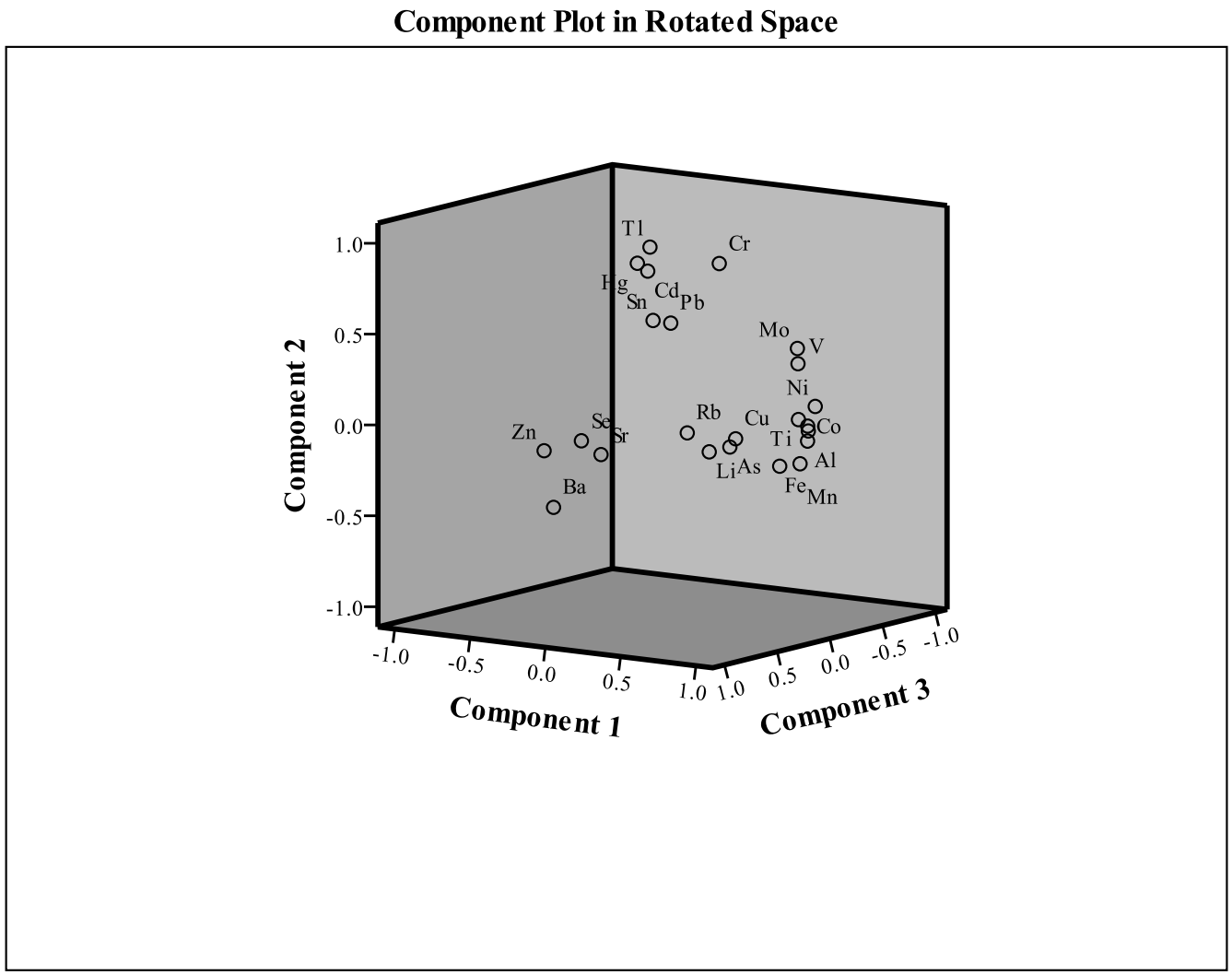

Figure 3.16: PCA component plot of all metal data across all sample types indicating groupings of the three components used to account for the variance in the data.

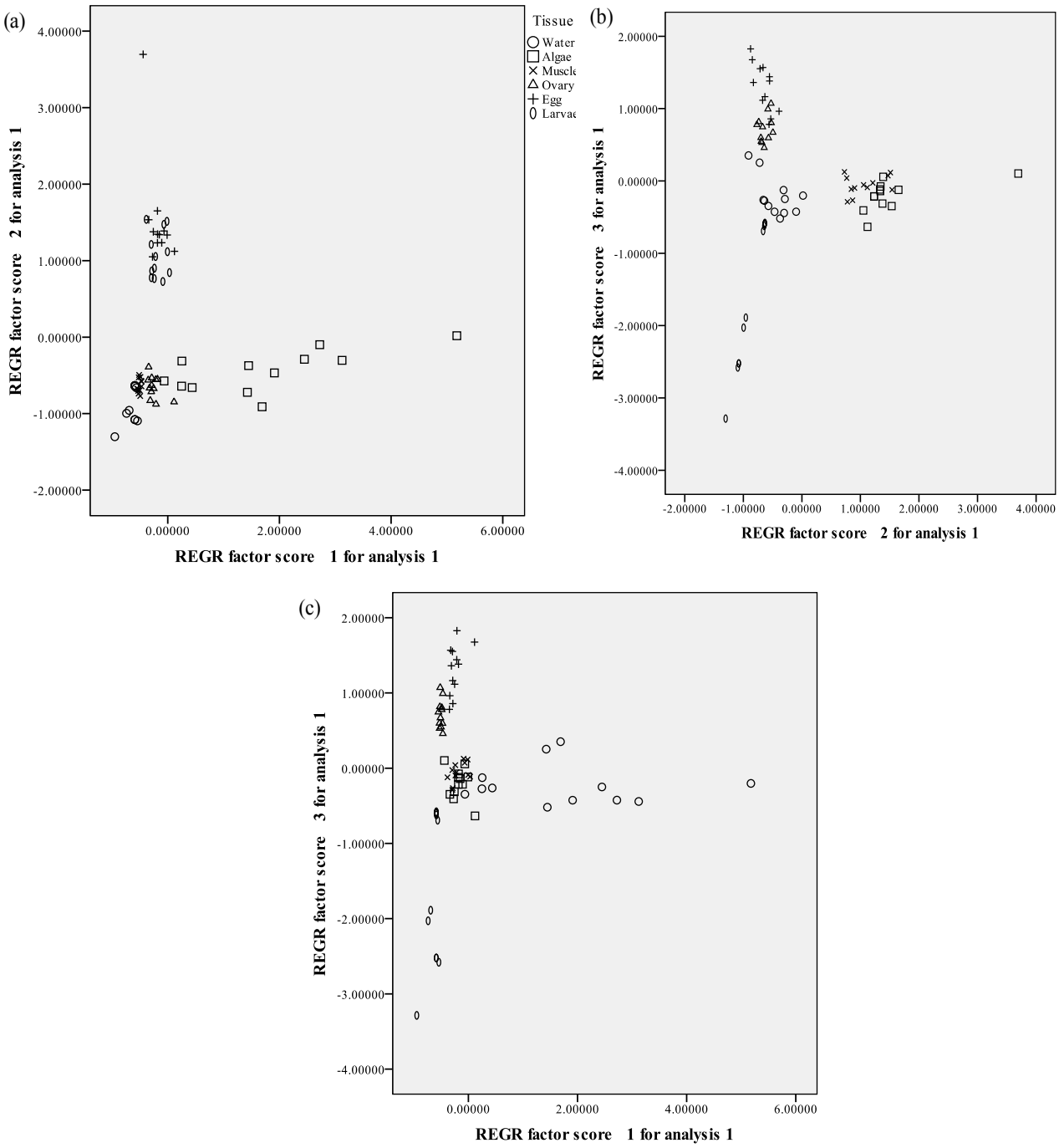

Figure 3.17: Scatter plots of PCA factor scores based on sample type (water, algae, muscle, ovary, eggs or larvae) following analysis for all metals data: a) factor score for Components 1 and 2 b) factor score for Components 1 and 3 c) factor score for Components 2 and 3. Where REGR refers to the regression factor score assigned to the raw data during PCA.

suggest that the water has an influence on the algae and that algae is the link between the water and the adult (muscle and ovary) tissues and offspring (eggs and larvae) tissues. Again it must be remembered that this is for all metals measured in the experiment and not limited to Se, where the metals most strongly loaded in each component will be driving what is visualized in the component plots (Figure 3.17a-c).

Additional PCA assessments were run where the biological data and Rb and Se data were included. The results of that analysis indicated that the strongest loading components were Rb in water (0.993) and egg size (-0.973) as well as Se in larvae (0.913) (Table 3.8). Examination of the loading plots indicate that number of spawns and % larval deformities loaded most closely to Rb in muscle tissue. E/f/d loaded closely to both Rb and Se in egg tissue. Egg size loaded closest to % hatching success and % larval survival though did not load close to Rb or Se in any of the evaluated matrices (Figure 3.18).

3.3 Discussion and Conclusions

The results of the correlation analysis techniques have provided support for our initial conclusions that in this experiment there was a very strong water mediated effect based on observed results of little interaction or contribution from site sediments (Driessnack et al. 2011a). This is visually demonstrated best in Figure 3.13, where the RW treatments are grouped together with no distinct sediment ordination and the same was seen with the grouping of the EW treatments also with no clear trend based on sediment type. The best correlation relationship was observed for Se in the water and algae which was expected as the algae/biofilms are suspected to be a primary contributor in the movement of dissolved Se from the water into the food web. Similar findings have been reported with Se in water and zooplankton by Hamilton et al. (2005a) who reported an $r = 0.90$ similar to ours of 0.927. Providing support for this there is a strong relationship between Se entering water and concentrations observed later in the primary producers, which will contribute to Se uptake as it moves to higher trophic levels. This compartment is requiring special consideration when evaluating Se in an impacted system.

We did not however see equally strong correlations between algae and the fish muscle and ovary Se levels as expected if diet was the sole source of Se in this experiment. This in itself is an important result as uptake of Se into the fish is complex and most likely originates from

Table 3.8: Loading for selenium (Se) and rubidium (Rb) in the different matrices (water, algae, muscle, ovary, egg and larvae) and biological endpoints. Numbers closest to +/- 1 loaded the strongest in each component

	Component 1	Component 2
Se Water	0.887	-0.420
Se Algae	0.877	-0.430
Se Muscle	.0900	0.134
Se Ovary	0.899	0.218
Se Egg	0.867	0.053
Se Larvae	0.913	0.124
% Larval Deformities	0.703	0.435
Egg Size	-0.973	-0.011
% Hatching Success	-0.719	0.076
% Larval Survival	-0.744	0.337
Number of Spawns	0.763	0.427
Eggs/female/day	0.859	0.002
Rb Water	0.993	-0.016
Rb Algae	0.636	-0.690
Rb Muscle	0.710	0.548
Rb Ovary	0.678	0.235
Rb Egg	0.852	0.021
Rb Larvae	0.404	-0.421

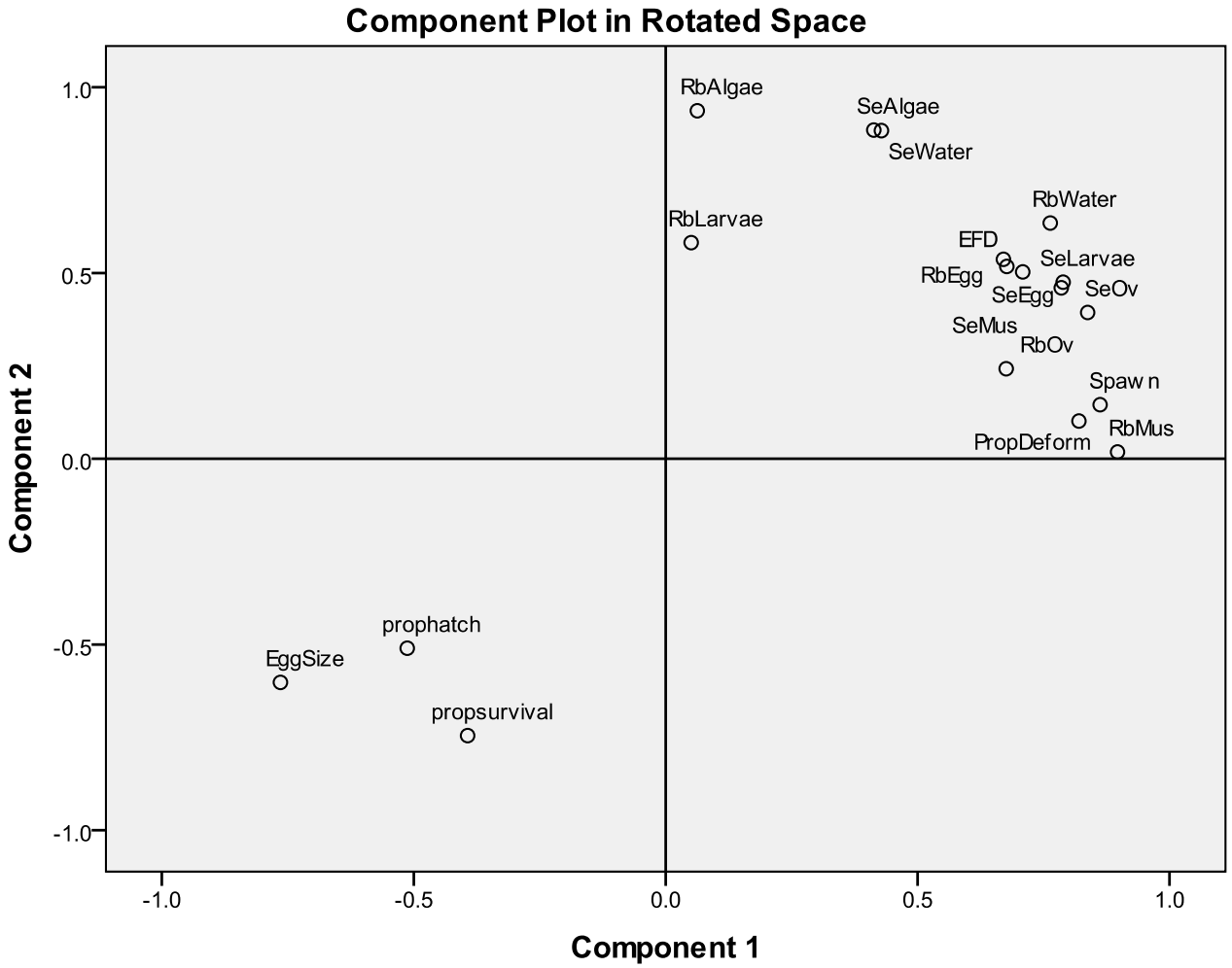

Figure 3.18: PCA component plot of for all selenium (Se) and rubidium (Rb) data in water, algae, muscle (Mus), ovary (Ov), egg and larvae as well as whole body Se (wholebody), eggs/female/day (EFD), number of spawns (Spawns), percent fertilization (PropFert), percent hatching success (prophatch), percent larval survival (propsurvival), percent larval deformities (PropDeform) and egg size as diameter (EggSize) are represented all sample types indicating groupings of the two components used to account for the variance in the data

multiple sources: water, diet (algae/biofilm and invertebrates) and sediment. The major contribution was from the diet, however the other sources cannot be entirely excluded. Although sediment did not contribute largely to observed results, sediments from the same lakes at the Key Lake site with higher TOC content contained higher amounts of Se and are likely to be ingested by small-bodied fish while foraging for invertebrates (Wiramanaden et al. 2010a,b). In relation to other studies, previous correlation values of r = 0.74 and 0.65 have been reported for Se in muscle and water and zooplankton respectively (Hamilton et al. 2005a), which are very similar to values we observed for Se in water and muscle. These results support Se incorporation into fish tissues being ultimately influenced by multiple sources and not a single defined factor.

In terms of the exchange of Se between fish tissues and offspring the strong correlations observed were expected based on values previously reported (Tables 3.1 and 3.2). In terms of egg and adult concentrations other studies have examined Se in eggs in relation to Se in muscle tissue and muscle plugs with r = 0.81 and 0.85 respectively being reported for razorback suckers, r = 0.864 for rainbow trout and r = 0.954 for brook trout (Hamilton et al. 2005b, Holm et al. 2005). In the mesocosm work we observed a similarly strong correlation. However our reported coefficient of determination is lower than those reported by Muscatello et al. (2006) and Muscatello and Janz (2009) for egg and muscle of r^2 = 0.83, 0.60, 0.98 for northern pike and spottail shiner. This may be attributed to different species sensitivities or duration of exposure, as the sample size used were both n = 3 per treatment.

The mesocosm work also allowed for comparison between Se in eggs and ovaries, where a strong correlation was observed as well as between larvae and ovary. In ovaries and egg/larvae we see the expected strong correlation due to the maternal transfer potential of Se (Schultz and Hermantuz 1990). Observed correlations and regression between Se in eggs and larvae were strong, suggesting that Se in larvae originated primarily from the egg with limited uptake from the water, as the larvae were not fed prior to collection, so dietary uptake of Se was considered to be negligible. Further examination showed a strong correlation between % larval deformities and egg Se levels, although with a moderate r^2. From this it could be concluded that in the mesocosm work a relationship exists between egg Se and deformities yet due to the lack of a coefficient of determination (r^2) greater then 0.500, there may be other factors influencing either variable that if accounted for in a multiple regression model may improve the observed value. However, it must

be remembered that the fish were exposed to a complex effluent with elevated Se. A previous evaluation of Se in eggs to % larval deformities was carried out by Muscatello et al. (2006) who also identified relationships between individual types of deformities (edema, craniofacial, skeletal and finfold) and total observed deformities with strong r^2 values of, for example, r^2 = 0.82 for egg Se and % total deformities. These authors also evaluated muscle Se and % total deformities with an r^2 of 0.80, where we indentified rho = 0.80 and r^2 = 0.413 as well as rho = 0.822 and r^2 = 0.574 for Se in larvae and % total deformities. Indicating that our observations of a relationship between deformities and egg Se is in agreement with work reported in the literature for field collections from the Key Lake site. Hamilton et al. (2005b) also evaluated Se in eggs and % total deformities (r = 0.27) and Se in muscle and % total deformities (r = 0.55). Additionally, the metal PCA also supports the strong linkages between muscle and ovary as well as eggs and larvae due the ways in which the different tissues loaded on the component plots.

Also of note are the relationships between egg size and Se in the ovary and eggs. Both ovary and eggs Se strongly and negatively correlated to egg size, indicating that as Se increased there were decreases in egg size measured as diameter. Egg diameter has been evaluated by Hamilton et al. (2005b) in relation to Se in muscle plugs where a strong correlation of r = -0.80 was reported. When we compare this with the mesocosm work Spearman rho = -0.866 for muscle and egg size the values are similar. This provides support for the need for increased consideration of egg size as a possible indicator of reproductive output. The strong egg size correlations were expected based upon findings reported in the literature for effects related to egg size. It has been reported that reduced egg size can have effects on the fitness of the resulting larvae (Einum and Fleming 1999, 2000, 2002). This is seen for instance with the correlation between egg size and percent survival, with survival decreased as egg size is reduced. These two endpoints were also grouped together in the PCA plot, further providing support for their strong relationship. This was reported also by Einum and Fleming (1999, 2000) where increased egg size had a positive effect on post- hatch success of larvae in terms of their survival, size and growth. Correlations were observed between egg size and Se levels in the eggs and larvae (rho = -0.848 and -0.817) with fairly strong coefficients of determination (r^2 = 0.675 and 0.77). The values for egg size and egg Se was considerably greater than those reported by Hamilton et al. (2005b) of r = -0.22. We can also compare further with Hamilton et al. (2005b) in terms of % hatch, % larval deformities and % survival in relation to egg size where the values they reported

were r = 0.41, -0.40 and 0.45. These are again lower than those reported here of rho = 0.537, -0.770 and 0.628. Comparison was also possible between egg Se and % hatch, % larval deformities and % survival for both studies: Hamilton et al. (2005b) r = -0.11, 0.27 and -0.17 respectively and the mesocosms rho = -0.497, 0.809 and -0.736. Overall these results suggest a possible relationship among egg size, and egg Se and offspring status.

It is hard to determine any completely clear relationship between Se in the tissue and resulting egg size therefore further examination into causes of changes in egg size and how this relates to Se should be considered. Maternal diet and CF have been reported to influence egg size, in that females can modify egg size as a response to environmental influences and changes in CF in females following exposure to Se (Einum and Fleming 2002; Kumuran et al. 2007; Driessnack et al. 2011a,b). Se and egg size being related is most likely a result of altered nutrient status in the female which can reduce the amount of nutrients deposited into yolk of the egg. However recent studies have also demonstrated changes in gonadal steroidogeneis due to exposure to Se-Met (Wiseman et al. 2011). It is possible that altered hormone levels could alter egg production rates and egg size. In addition larger brood size will also result in more sharing of available nutrients among all the eggs. Related to the yolk is vitellogenin, the yolk precursor, which is during the production of vitellogenin where Se-methionine would be incorporated and eventually comprises a large portion of the yolk (Kroll and Doroshov 1991). In this context it is important to note that *P. promelas* vitellogenin is relatively richer in methionine, 2.6% of residues vs. 1.8% average vertebrate methionine frequency (Korte et al. 2000). Therefore, we might expect vitellogenin to be relatively rich in Se-methionine when adults are exposed to Se. Thus, a combined effect of reduced nutrient status of the female and increased opportunities to incorporate more concentrated (due to reduced available nutrients) Se-methionine into the yolk via vitellogenin may have contributed to reduced egg size and required the developing larvae to rely upon yolk with suspected greater amounts of Se-methionine which contributed to increased incidences of larval deformities (Kroll and Doroshov 1991; Rennie et al. 2005; Hamilton et al. 2005). It has also been noted that a critical time for the production of high quality eggs is during vitellogenesis, therefore if Se is affecting this process one of the impacts could be manifest as decreased egg size (Navas et al.1997).

The Key Lake effluent is a complex uranium milling effluent and Se only loaded strongest in the third component in the PCA metal evaluation. Therefore the results can support the hypothesis that Se is a contributor to the observed effects in this system, but the potential for other metals to have similar, antagonistic or synergistic effects cannot be disregarded. It was also indicated in the PCA that Se did not load until the third component and then not as strongly as other metals. Se proportion accounted for in component 3 was 0.672 compared to Zn at 0.805 and As of -0.756 in the same component, where As is also known to interact with Se. In addition Cd and Ni loaded stronger then Se; Cd was 0.819 in component 2 and Ni was 0.954 in component 1. Both metals may also have effects on egg endpoints (Pickering and Gast 1972, Dave and Xiu, 1991, Gauthier et al. 2006). Again, some metals such as Rb were found to be elevated in all tissues and are known to bioaccumulate in aquatic systems, yet no information is currently available on their impacts on egg production or egg/larvae health and success (Campbell et al. 2005).

In regard to Rb, as was shown with the additional correlation and PCA analyses, interesting results were observed. Firstly, when examining the correlation between Rb in water versus Se in water, stronger correlations were noted between egg size and Rb (rho = 0.-856) compared to Se at -0.796 though both were highly significant. This was also seen in regards to the percent larval deformities with Rb (rho = 0.842) and Se (rho = 0.728), and e/f/d with Rb (rho = 0.876) and Se (rho = 0.690). One of the most interesting correlation was number of spawns, were between Rb in water (rho = 0.893) was highly significant, and Se in water (rho = 0.487) was not significant. From this it would suggest that Rb based effects may be more strongly water based versus Se that is dietary based, where Rb in algae did not correlate as strongly with any of the endpoints when compared to Se in algae. Other comparisons of interest were Rb in larvae tissue when compared with percent hatching success (rho = -0.845) compared to Se in larvae with rho = -0.614. As well as Rb in egg tissue and percent larval deformities (rho = 0.815), which is quite comparable to Se in egg tissue (rho = 0.809).

When the PCA of Rb and Se levels in the different matrices and biological endpoints (Figure 3. 18), is examined it is interesting that Se, a known teratogen, did not load as close to percent larval deformities as would be expected. Though many of the points were all clustered in the same general area. Also interestingly, both Rb and Se in egg tissue were closely related to

e/f/d. Wiseman et al. (2011) recently reported increased ovarian steroidogensis following dietary exposure to Se-methionine. From the speciation data reported by Driessnack et al. (2011a) it was indicated that these exposed eggs had higher proportions of Se-methionine like compounds, which may suggest there was increased steroidogensis and may be why e/f/d and egg Se loaded so closely. It also raises the question of how Rb in egg tissue is possibly involved. These findings would suggest that Rb may have an effect on reproduction in FHMs and should be examined further.

The results related to spawning and e/f/d are unique to the mesocosm work and have not been noted in the literature reviewed here with respect to Se. The findings here indicate that the status of the adults as represented by Se in muscle and ovary tissues have a stronger correlation than the environmental matrices in terms of reproduction as well as for the first time with Rb. In the mesocosm work presented by Driessnack et al. (2011a) there was an increase in egg production following exposure to the uranium milling effluent at an environmentally relevant concentration, by diluting whole effluent to match concentrations in the exposure lake, Unknown Lake. This has been attributed to a possible hormetic effect due to the effluent being higher in nutrients then the reference lakes at the site. Therefore, it is possible to conclude here that reproductive output is affected by exposing adults to effluent elevated in Se, and that effects on egg size, offspring status and uptake are all related to these changes in egg output. The extent of this relationship is not fully defined here and would need to be evaluated with a dose response relationship to better identify trends.

The results in the above analysis can be viewed as a tool to allow for higher resolution visualization of Se. Relationships between Se in the water and primary producers are the strongest and indicative of the importance of the lower trophic levels making Se bioavailable to the much more sensitive fish as well as aquatic birds. So what does this mean in terms of environmental regulation? Since low levels of aqueous Se have been shown to cause reproductive toxicity in fish, it means there is a strong `species to species' variation in sensitivity that needs to be addressed. In terms of what form of Se is entering the system and what species of primary producers are in the system, it must be considered whether they are reducers or do they accumulate Se far beyond requirement. This has been explained in further detail in a site summary for the Key Lake facility upon which the mesocosm work was based (Driessnack et al.

2011a, and Driessnack et al. draft). The results also suggest that egg size is an endpoint to consider in future reproductive work and not just in relation to Se. Egg size is an endpoint required in EEM programs for Environment Canada and has been reported for other industrial effluents, not just those containing Se (Driessnack et al. 2011a, b).

It is the goal of this research to better define the Se species uptake and egg size question through further FHM reproductive bioassays. Some work has already commenced and pending completion of the data analysis will be included with the current correlation and PCA techniques as well as future planned studies. This initial synthesis of other literature results allows for the identification of questions that could be studied so as to provide the greatest strength and support to the already vast collection of Se data.

Chapter 4:

General Conclusions

4.1 Project Rationale

Uranium mining is one of Saskatchewan's largest industries but represents only one sector of Canada's diverse mining industries. These industries are of great importance from an economic perspective, but they are also of great concern when assessing environmental change in receiving waters to which the effluents are discharged. Although all mines and mills are required to treat their effluent prior to discharge, the receiving environments are still monitored under the EEM program. This program has demonstrated a trend towards decreased condition factor, liver size and growth rate in fish and changes in invertebrate density and richness in impacted aquatic systems (Lowell et al. 2007). Some of these changes have been noted at the Key Lake uranium facility, where additional research has also identified Se as a potential contaminant of concern (Klaverkamp et al. 2002; CNSC 2006; Muscatello et al. 2006, 2008).

Therefore my goal was to better understand Se in this system by using field-based mesocosms (a method approved for fish health evaluation under EEM) using water and sediment exposure routes in a fully factorial design. This was achieved by assessing biological, reproductive and tissue burden endpoints in fathead minnows exposed to conditions found in the affected receiving environment. In addition, advanced Se speciation techniques permitted better evaluation and understanding of the effects of Se specifically while still considering whole effluent effects.

4.2 Constituents of Potential Concern

Although the focus of this work has been on Se, other metals have been noted to be increased in the effluent, algae and fish tissues. One such metal to consider further is rubidium (Rb). Rb was found to be significantly elevated in all tissues measured here as well as increases have been noted by Rozon-Ramilo et al. (2011). Rb has been identified as having the potential to bioaccumulate in both freshwater and marine aquatic systems (Campbell et al. 2005). In this work Rb did not bioaccumulate in the system but we did have levels in the FHM muscle similar to those reported for fish collected from Lake Erie; Lake Erie 1.7-3.3 µg/g w.w and exposed mesocosm FHMs 4.40-4.93µg/g ww (Campbell et al. 2005). There has been some work on Rb in regards to reproduction, where decreased spermatogenesis in fish was noted in testes with concentrations of 18 µg/g d.w (Yamaguchi et al. 2007). Although we did not analyze male

tissues in this study we did note Rb levels in exposed FHM female muscle and ovaries at 22-25 µg/g d.w and 24-26 µg/g d.w respectively assuming the same 80% moisture as Yamaguchi et al. (2007). In regards to Rb and the mesocosm work it is a metal that should be considered further as little is known about the effects of the metal alone or in a complex effluent. Also of note is that one potential source of Rb is from coal and fly ash, which is also a well noted potential source for Se.

As was discussed in Chapter 3, additional statistical analysis (correlation and PCA) have indicated strong relationship between observed effects and Se but also Rb. Both metals correlated negatively, strongly (rho $\geq$ 0.700) and significantly with their respective water concentrations and egg size. Also of note was a very strong and significant correlation between number of spawns for correlation with Rb in water which was not noted for Se in water. Also of interest was Rb and Se levels in egg tissue had very similar correlation values when compared with percent larval deformities. When the PCA loading plot for the Se and Rb data was examined it was indicated the Rb in larvae loaded closest to percent larval deformities. Examination of egg tissue levels for both Rb and Se showed both loaded very closely to e/f/d. This would suggest that Se is a driver in the system but since such strong correlations were also noted for Rb it is hard to deem it the sole causal factor.

Molybdenum (Mo) was also noted to be increased and has been of concern at the site as well. Pyle et al. (2001) noted in the results of PCA that Mo and larval mortalities were strongly loaded on Mo, yet laboratory work using similar concentrations did not elicit the same effects. Other metals to consider are Cd and Ni, as they may have potential effects on time to hatch in eggs and should also be considered further especially in how they may relate to changes in egg size. Ni has also been observed as a contaminant of concern for uranium mining activities (Dave and Xiu 1991; Pyle et al. 2001, 2002a). Early life stages are most sensitive to Ni, although increased hardness appears to reduce toxicity (Pyle et al. 2002b). Effects have been observed at both the cellular and sub-cellular level in fathead minnows following exposure to Ni at 16 µg/L in water and 10 µg/g in the diet (Lapointe and Couture 2009). Water levels in the mesocosm work were 32-35 µg/L Ni and could have possibly also affected the fish and offspring health; however our potential dietary exposure through the algae/biofilm was much less at 3 µg/g d.w. The effluent is also characterized as being elevated in ammonia, hardness and conductivity.

Ammonia can have impacts on the survival of fish but may also affect reproduction at low levels (Thurston et al. 1986). Hardness and pH can also affect the potential toxicity of the metals in the effluent by reducing their availability. Hardness may have the potential to influence skeletal development and growth in FHMs, where some differences in survival, body mass and caudal fin ossification in low (< 50 mg/L) and high (> 175 mg/L) hardness (Blanksma et al. 2008). These values are much less than in our exposure treatments (455-463 mg/L) but hardness could be involved in the observed effects as not all can solely be contributed to Se.

4.3 Recommendations

Based on the results of these studies, more attention needs to be paid to the trophic transfer of Se in the aquatic ecosystem. Work also needs to be done to more clearly define if Se is the main contributing factor to adverse effects in this system. The results presented here showed elevated levels of other constituents, some of which are known to interact with Se in organisms as well as its uptake (Hg, As, sulphate). Although a weight of evidence approach previously identified Se as the causal contaminant in the system, the interaction with the other components on uptake needs to be considered further. As the uptake at the algae/biofilm level appears to be the driver for Se transfer to other trophic levels this research suggests that more work on the uptake and transformation of Se through the biofilms is required to elaborate the mechanisms of transfer to invertebrates and fish. If this can be better quantified the process of developing Se guidelines will be eased. The redox environment should also be characterized and the species in the algae/biofilms identified. Both of which lead back to the "species species" question for Se uptake; by knowing what form or species of Se you are dealing with and how the species of primary producers present transform Se, then thresholds should be easier to define. Or more simply thresholds should be defined when the species of Se in the system and how the species of organisms impacted interact with each other are identified.

In terms of reproduction the changes in egg size is an endpoint that warrants further attention. This endpoint alone was correlated with the success of the offspring and may be used as an indicator in other systems. It has been noted in other work with industrial effluents that egg size is reduced following exposure (Weber et al. 2008; Rozon-Ramilo et al. 2011). Since egg size can be manipulated by the females in response to dietary and environmental conditions, this endpoint may be a valuable indicator of the often more susceptible early life stage fish status in a

system (Einum and Fleming 2002). Failure to replace breeding adults with healthy offspring that will be able to reproduce is vital for a population to persist. If a certain percentage change in egg size can be linked with other endpoints such a percent changes in mortality and deformities this would be an endpoint of considerable value since it could potentially be used to assess impacts in naturally exposed fish populations in the system. Another aspect to consider is what implications the numbers of normal larvae have on potential population level effects. Examination of the mean total indicated no differences between treatments where as proportion did indicate a significant difference. It is essentially one endpoint with two possible implications on population. The meaning and strength of this endpoint should be evaluated further, such as in relation to the US EPA (2004) suggestion of a 20% threshold level (EC20) for Se mediated effects. However, it is important to note there is a growing shift towards use of 10% or EC_{10} in regards to Se thresholds and both values should be determined. Again in this work we saw decreased larval survival, hatching success and egg size but those decreases were offset by the increased reproductive output. Which lends the question: are the females in the diluted effluent sacrificing some of their offspring's fitness for increased fecundity and is it a successful trade off and for how long? Is this the generalized phenomenon that some highly fecund organisms sacrifice their offspring survival in favor of maternal reproductive fitness as suggested by Einum and Fleming (2000)?

In regards to speciation work using synchrotron technology, it's a positive step in the goal of understanding Se yet it is not without its own complications. Although the data provided from synchrotron speciation is valuable and detailed there is a lack of replication, therefore all that can be reported is suspected trends from the speciation spectrum with no statistics to support those conclusions. We currently cannot statistically test if there is a significant difference in the proportion of selenium species observed in samples and add strength to the conclusions drawn. This lack of replication stems from the cost of running these samples and limited beamtime availability. When an allotted 12 hr beamtime period may only result in 10-12 samples being analyzed it may take months or years to obtain the required results. Therefore the move to less expensive and faster methods for speciation is ideal and exists potentially in HPLC-ICP-MS methods. Where the HPLC portion separates the Se forms or species and then detected in the subsequent ICP-MS portion of the analysis. The benefits of being able to analyze more samples

with a much quicker turnaround time allows for adequate replication and allows for a larger amount of data to be integrated into the current Se knowledge-base.

The sediment effect in this work was not a huge driver but is not a factor that should be entirely excluded from consideration as Cameco Corporation moves forward with the Key Lake facility. Sediment can serve as a pool of Se for many years that may initially not be available but could be mobilized from changes in water quality that may alter the porewater redox environment but also from invertebrates and primary producers. These alterations or changes could potentially contribute to elevated levels of Se in the system for years after effluent discharging ceases. Of greatest consideration would be sediment with higher TOC content compared to the low TOC in the current work, both of which exist in the site lakes. So although not a major contributing pathway in the fish mesocosm work, it is an important pathway to consider for invertebrates that are a dietary pathway for fish.

In terms of the mesocosms there are some modifications that could be made to increase their efficiency. In particular using the manifold and march pump distribution system, the water entering the streams through this system is very fast flowing and not the best representative for lake systems. This could be substituted with a slower and easily adjusted flow rate delivered by a peristaltic pump system. This slower flow would have allowed for settling of high organic content sediment in the mesocosm systems in this work. Trials with this system for further work with sediment has shown it as a suitable alternative where sediment was able to settle but there was sufficient flow to maintain low ammonia levels in reference water streams with fish present in the streams. This would also allow for evaluation with higher organic sediment and lower organic sediment to evaluate trophic transfer into invertebrates such as *C. dilutus* if the mesocosm were used in a multi-trophic fashion as used by Rickwood et al. (2006a,b,c, 2008) and Rozon-Ramilo et al. (2011).

Therefore, based on this work it would be beneficial to still consider Se as contributing to the observed effects yet is not the sole basis for those effects. Again this reiterates that a complex effluent is being discharged and to suggest only one of 20 metals found to be elevated in the effluent and many of which were also detected in the fish muscle, ovaries and offspring as the entire causal factor seems unrealistic. This is not to discredit any of the prior work at the facility that has identified Se through weight-of evidence approaches, as the scope and goals of those

studies are different than the work presented here. This study was one of the first to attempt to quantify the reproductive implications on fish exposed to the effluent mixture, not Se alone. This is where further work to compare the effects between Se alone at levels in the diluted effluent and diluted effluent are compared to note if similar or different trends are observed.

4.4 Conclusion

This work allowed for the health, survival and reproduction of FHM to be characterized following exposure to environmentally relevant effluent and sediment concentrations exposed sediment in a fully factorial mesocosm application. This study was conducted to fill a gap due to limited studies on the detailed reproductive implications (e.g. egg production) for fish exposed to site conditions. The use of the 21-day partial lifecycle FHM reproductive bioassay provided insight not only into the incidence of larval deformities but also egg health and production and this was linked with the status of the exposed adults. Where egg health here being viewed as being reduced by decreases in egg size, hatching success and/or reduced time to hatch. Trends indentified during analysis of these endpoints suggested a primarily water-borne effect with little contribution from the sediment. This minimal sediment contribution has been attributed to the low TOC content in the sediment chosen for use in the study. The choice of this low TOC sediment was justified in that it was collected from the margin of the lakes, areas commonly inhabited by FHM adults, larvae and juveniles in ecosystems they inhabit. Although limited effects were noted the importance of sediment is still a point to be considered as Se concentrations have been linked to TOC content (Wiramanaden et al. 2010a). Therefore use of sediment with high TOC may have had different effects though we feel the choice of our sediment was justified as it was representative of sediment in the impacted lakes.

The adult morphometric endpoints primarily indicated a decrease in male liver size following water exposure and an interaction for female condition factor. Despite these changes egg production was significantly increased following exposure to 25% diluted effluent, representative of Unknown Lake. Although a larger number of eggs were produced following effluent exposure the quality of those eggs appears to have been inferior to those in the reference treatment. Eggs exposed to 25% effluent were significantly smaller in size and demonstrated reduced hatching success. Of the larvae to successfully hatch, those in exposed water displayed increased mortality and incidences of deformities. Further data analyses indicated that despite the

initial increase in the number of eggs the mean total number of apparently normal larvae per trio was comparable among all treatments. This has led to the suggestion that initial exposure to effluent stimulates egg production potentially as a hormetic response due to increased nutrients in the water compared to the nutrient deficient reference water. However, this positive response was not duplicated in the health of the eggs and led to the conclusion that Se or other components of the effluent are mediating the effect. In addition it was noted that exposure led to early hatch and many larvae were extremely lethargic, in terms of not swimming and remaining sedentary in the egg cups, and underdeveloped, in that they were much smaller in size, had very large yolk sacs and little development of features such as in the cranial region. In the natural environment these larvae would be highly vulnerable to predation and the survival rate and number of normal live larvae reported in the mesocosm study may be greater than would be observed in the actual lake.

Reproduction based endpoints were not the only endpoints evaluated in this study. Tissue concentrations were measured for female muscle and ovary as well as for eggs and larvae. Additional samples were taken for total Se in water, sediment and algae/biofilm in the streams. The results showed significant increases in multiple metals across all the samples from fish exposed to effluent, although no differences were noted for exposed solely to contaminated sediment. Se was increased in the water, algae/biofilms and in the fish tissues. These increases correspond with other reported data at the site and provide support for Se contributing to the observed reproductive effects. Again, this is a complex effluent and other metals were increased along with Se such as Rb, Cu, Cd, and Mo. Therefore to clearly identify Se as the primary causal factor is confounded by the nature of the effluent. Thus the inclusion of the Se speciation assessment in the project helped to provide more support for the key role of Se. By identifying the proportion of Se in each of the tissue samples we were able to identify shifts in Se speciation patterns and trends observed in reference samples.

The results of the speciation analysis can be best summarized as a shift in the proportion of selenocystine like compounds to selenomethionine like compounds following exposure. This shift in proportion is not unexpected and is supported by the literature which indicates that selenocysteine concentrations are highly regulated while selenomethionine represents an uncontrolled pool of excess Se (Suzuki 2005). Se is stored as a pool of selenomethionine to

which Se is actively scavenged from other cellular processes. Increases in the amount of Se in the body also influence a shift from Se being an antioxidant to a prooxidant. As the body transforms excess Se, greater amounts of ROS can be generated. The algal speciation was only possible for the exposed samples so comparison to reference was not possible but it was noted that the greatest variety of Se species were present in the algae. This result is very important as the water speciation showed primarily the inorganic selenate and selenite, whereas as in algae DMSeO, MSe, and GSe were identified. This indicates that, as has been suspected, the biofilm/algal component in impacted ecosystems is a driver in the transfer and transformation of inorganic Se from water into organic forms at higher trophic levels. This is important as selenate is not readily taken up by invertebrates and fish, so the primary producers are facilitating the transformation and uptake of Se in this ecosystem. This also suggests a significant if indirect role for sediments as these primary producers often live on the sediment surface and the redox potential in the sediment can contribute to the observed transformations. So although we did not find sediment to be a larger contributor in this research, it can be an important component in different aquatic ecosystems.

4.5 Scientific Contribution

The results of this project are valuable to the scientific community, regulators, industry and to Cameco Corporation as they further knowledge on the dynamics of Se at the Key Lake facility. The data has provided additional information on the effects of exposure to a complex mixed effluent on FHM health and reproductive success. Being able to thoroughly characterize the reproductive implications at the Key Lake facility had not been done before although larval deformities have been assessed previously in field caught specimens (Muscatello et al. 2006). The results support the contention that the algae/biofilm are a key component in Se movement through the system, and that use of water only exposure scenarios and guidelines are not appropriate for the management of Se. Although Se is a contributing factor in this system the complex nature of the effluent confounds our ability to clearly identify all contributing factors. In terms of reproduction the sensitivity of egg size is an endpoint that merits further consideration as in these results reduced egg size provided support for inferior egg quality and was correlated with reduced larval survival and the increased prevalence of deformities.

This project can be viewed as a critical bridge connecting previous laboratory based and field based studies at the Key Lake facility by focusing on Se in a cohesive project. This project also serves as a stepping stone to additional mesocosm work that may choose to incorporate a specific algal/biofilm component in trophic transfer mesocosms as employed by Rickwood et al. (2008), to fully address the dynamic nature of Se. The data can also continue to be integrated with other correlation and regression work in the literature to identify and characterize the strength of relationships between fish and Se from various angles. Another advantage of this work is that the results from the integrative project can be developed into a model that could ideally be used to set guidelines for Key Lake or used as a template for other uranium mining and milling facilities. In addition, other industries, especially coal related activities, another major source of selenium, will continue to grow as demands for energy also expand where the result here could be of use in their monitoring programs.

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Appendix A: Table 1: Total metal and composition analysis of reference water (RW) and 25% effluent (EW) in the presence of either reference sediments (RS) or contaminated sediments (CS) after 21-days in mesocosm streams. Three samples taken from each treatment following exposure. Values are mean (n =3) ± standard error of the mean.

Variable[1]	RWRS	RWCS	EWRS	EWCS
Hg	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Al	360.00 ± 68.07	680.00 ± 134.82	273.33 ± 147.12	663.33 ± 15.28
SB	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
As	0.50 ± 0.06	0.57 ± 0.06	0.43 ± 0.06	0.60 ± 0.03
Ba	5.33 ± 0.87	6.60 ± 0.71	3.73 ± 0.77	5.10 ± 0.50
Be	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Bi	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Bo	0.50 ± 0.00	0.50 ± 0.00	1.33 ±0.00	2.00 ± 0.00
Cd	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Cr	113.97 ± 113.02	1.13 ± 0.06	0.77 ± 0.07	1.37 ± 0.15
Co	0.33 ± 0.23	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
Cu	2.07 ± 0.42	0.52 ± 0.32	1.00 ± 0.32	0.45 ± 0.14
Fe	1040.00 ± 880.17	440.00 ± 94.93	150.00 ± 106.82	610.00 ± 11.55
Pb	0.87 ± 0.09	0.87 ± 0.09	0.97 ± 0.06	0.80 ± 0.03
Li	0.47 ± 0.03	0.67 ± 0.09	0.47 ± 0.10	0.73 ± 0.03
Mn	9.43 ± 6.29	10.37 ± 1.98	1.80 ± 3.03	16.00 ± 1.32
Mo	0.53 ± 0.48	0.17 ± 0.05	0.20 ± 0.04	0.77 ± 0.03
Ni	2.57 ± 2.32	0.37 ± 0.09	0.40 ± 0.09	0.43 ± 0.07
Rb	0.43 ± 0.07	0.77 ± 0.12	0.33 ± 0.13	0.63 ± 0.07
Se	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Ag	0.05 ± 0.00	0.05 ± 0.00	0.05 ±0.00	0.05 ± 0.00
Sr	21.33 ± 1.86	19.33 ± 1.86	20.33 ± 0.33	19.33 ± 0.33
Tl	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
Sn	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	20.20 ± 0.00
Ti	18.00 ± 3.46	41.67 ±8.19	12.67 ± 10.59	38.47 ±
U	0.23 ± 0.03	0.23 ± 0.03	0.37 ± 0.03	0.53 ± 0.03
V	1.13 ± 0.88	0.60 ±0.12	0.47 ± 0.13	0.55 ± 0.00
Zn	0.95 ± 0.42	2.85 ± 1.18	5.17 ± 1.30	2.28 ± 1.30
% Inorganic Carbon	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
% Total Organic Carbon	0.05 ± 0.00	0.07 ±0.02	0.07 ± 0.02	0.07 ± 0.02
% CaCO3	0.97 ± 0.03	0.80 ± 0.00	0.83 ± 0.03	0.93 ± 0.07
% Gravel	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
% Coarse Sand	95.67 ± 0.33	59.00 ±6.03 S	94.33 ± 0.33	58.33 ± 0.88 S
% Fine Sand	3.67 ± 0.33	39.33 ±5.84 S	4.67 ± 0.33	40.00 ± 1.15 S
% Silt	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.67 ± 0.17
% Clay	1.00 ± 0.00	1.33 ±0.33	1.00 ± 0.00	1.00 ± 0.00

W indicates a water effect, S indicates a sediment effect and I indicates and interaction, where $p \leq 0.05$. Analyzed using a two-way ANOVA

1- all units are ug/g wet weight unless otherwise indicated
%- percent of sediment composition

Appendix B: Spearman Rank Correlation Coefficients for comparisons of selenium levels in the adult and egg/larvae tissues and egg size and biological effects. Where a p-value of less than or equal to 0.05 is considered to be significant.

		Se Water	Se Algae	Se Muscle	Se Ovary	Se Egg	Se Larvae	Egg Size	% Larval Deform	% Larval Survival	% Hatch	Spawns	E/F/D	TTH	MNL
Se Water	rho	1													
	p-value	-													
Se Algae	rho	0.902	1												
	p-value	< 0.001	-												
Se Muscle	rho	0.666	0.752	1											
	p-value	0.018	0.005	-											
Se Ovary	rho	0.614	0.729	0.865	1										
	p-value	0.034	0.007	< 0.001	-										
Se Egg	rho	0.737	0.755	0.836	0.768	1									
	p-value	0.006	0.005	0.001	0.004	-									

Appendix B Continued															
Se Larvae	rho p-value	0.753 0.005	0.756 0.004	0.717 0.009	0.870 < 0.001	0.846 0.001	1 -								
Whole Body Se	rho p-value	0.614 0.034	0.729 0.007	0.865 < 0.001	1.000 < 0.001	0.768 0.004	0.870 <0.001								
Egg Size	rho p-value	-0.751 0.005	-0.799 0.002	-0.871 < 0.001	-0.768 0.004	-0.844 < 0.001	-0.809 0.001	1 -							
% Larval Deform	rho p-value	0.739 0.006	0.715 0.009	0.786 0.002	0.768 0.004	0.810 0.001	0.824 0.001	-0.781 0.003	1 -						
% Larval Survival	rho p-value	-0.708 0.10	-0.829 0.001	-0.785 0.003	-0.715 0.009	-0.736 0.006	-0.607 0.037	0.643 0.024	-0.611 0.035	1 -					
% Hatch	rho p-value	-0.644 0.024	-0.702 0.011	-0.642 0.024	-0.635 0.026	-0.514 0.088	-0.620 0.031	0.572 0.052	-0.576 0.050	0.725 0.008	1 -				

Appendix B Continued															
Spawns	rho	0.460	0.546	0.701	0.833	0.768	0.854	-0.768	0.656	-0.576	-0.509	1			
	p-value	0.133	0.066	0.011	0.001	0.004	< 0.001	0.004	0.021	0.50	0.091	-			
E/F/D	rho	0.689	0.788	0.732	0.718	0.757	0.785	-0.777	0.775	-0.699	-0.585	0.760	1		
	p-value	0.013	0.002	0.007	0.009	0.004	0.003	0.003	0.003	0.011	0.046	0.004	-		
TTH	rho	-0.095	0.041	-0.209	0.121	-0.188	-0.058	0.250	-0.007	-0.014	-0.263	0.043	-0.077	1	
	p-value	0.770	0.899	0.515	0.708	0.559	0.857	0.434	0.983	0.966	0.409	0.895	0.812	-	
MNL	rho	0.160	0.101	0.067	0.049	0.386	0.218	-0.147	0.357	-0.056	0.385	0.192	0.399	-0.294	1
	p-value	0.620	0.755	0.837	0.880	0.215	0.496	0.648	0.254	0.863	0.217	0550	0.199	.0353	-

% Larval Deform – percent larval deformities

% Larval Survival – percent larval survival to 5-days post hatch

% Hatch – percent hatching success

Spawns- number of spawning events

E/F/D – eggs/female/day

TTH- Time to hatch

MNL- Mean number of Normal Larvae per trio of breeding fathead minnows